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Full Length Research Paper

Inter simple sequence repeat polymorphism in *Alternaria* genomic DNA exposed to lethal concentrations of isothiocyanates

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Isothiocyanates (ITCs) is a group of defense related compounds synthesized by *Brassicas* that have positive effects on human health which suggest that they are environmentally friendly compounds to control fungi infections. However, the development of ITC-resistant strains of fungi is a matter of concern. The objective of the present work was to study the response of *Alternaria alternata* to allyl-isothiocyanate (AITC) and benzyl isothiocyanate (BITC) chronic exposure and the effect of the treatment on some of the inter simple sequence repeats (ISSR) regions. Five strains of *A. alternata* isolated from tomato and five isolated from cabbage were independently exposed *in vitro* to AITC and BITC. Concentrations were increased until it reached 0.08 and 0.6 mg/mL of AITC and BITC, respectively. Genomic DNA from both wild type and isogenic adapted strains to ITCs was isolated and five primers were used for ISSR amplification: $(GACA)_4$:5´-GACAGACAGACAGACA-3´; M13:5´-GAGGGTGGCGGCGGTTCT-3´;$(AAG)_8$:5´-AAGAAGAAGAAGAAGAAGAAGAAG-3´; $(ACA)_5$: 5´-ACAACAACAACAACA-3´ and T3B: 5´-AGGTCGCGGGTTCGAATCC-3´. A lower degree of polymorphism was found to be induced by the treatment in the wild-type strains isolated from cabbage as compared with the wild-type strains isolated from tomato. It can be concluded that ITCs exposure induced random mutations in different ISSR regions of the *A. alternata* genome which does not lead to the development of strains with a hereditable resistant phenotype.

Key words: *Alternaria alternata*, fungicide, allyl-isothiocyanate, benzyl-isothiocyanate, inter-simple sequence repeat.

INTRODUCTION

Fungi in the genus *Alternaria* are commonly found in most ecosystem and are ubiquitous agents of decay. Numerous citations report recovery of species from such diverse substrates as sewage, textiles, stone monuments, cosmetics, computer disks, and jet fuel, and their role in the degradation of manufactured products

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causes large commercial losses (Rotem, 1994). In agricultural systems, *Alternaria* spp. are notorious postharvest pathogens and prolific producers of carcinogenic and teratogenic secondary metabolites (Woody and Chu, 1992). As such, the occurrence of *Alternaria* metabolites in processed foods and fresh fruits and vegetables is becoming an increasing environmental concern (Bottalico and Logrieco, 1998). In addition, these fungi are important primary and secondary plant pathogens that infect a wide variety of crops and cause considerable economic losses worldwide (Farr et al., 1989; Snowdon, 1991).

In agricultural systems, *Alternaria* and other fungal plant pathogens are controlled mainly through the use of synthetic fungicides. However, prolonged exposure to such chemicals often results in the development of fungal strains resistant to single or several fungicides (Baraldi et al., 2003; Nakaune et al., 1998). In many cases, the development of resistance is due to single mutations in the fungal genome that are heritable and subsequently selected for in the fungal population due to the selection pressure imposed by fungicide exposure (Albertini et al., 1999; Báez-Flores et al., 2008; Gisi et al., 2002; Yoshimi et al., 2005). This recurrent problem had prompted the search for alternatives to synthetic fungicides in an effort to prolong efficacy of disease management strategies (Troncoso-Rojas and Tiznado-Hernandez, 2007).

Promising alternative to synthetic chemical fungicides include naturally produced isothiocyanates (ITCs), a group of defense related compounds synthesized by plants of the genus *Brassica* (Fahey et al., 2001; Tiznado-Hernández and Troncoso-Rojas, 2006). Evidences had shown that these compounds have a strong antifungal activity against *Alternaria* and other phytopathogens under *in vitro* conditions (Manici et al., 1997; Rosa and Rodrigues, 1999; Tiznado-Hernández and Troncoso-Rojas, 2006), as well as *in planta* during fruit infection (Troncoso-Rojas et al., 2005a). In comparative studies, Mari et al. (1996) and Troncoso et al. (2005b) demonstrated that naturally obtained ITCs perform significantly better in preventing postharvest infection of mature fruit than several commercial fungicides.

Although ITC's offer a very promising alternative to synthetic fungicides in controlling fungi infections during postharvest, the possibility of development of resistance to ITCs in the fungal population is still a matter of concern. Indeed, preliminary studies in our laboratory revealed that *Alternaria* could become adapted to grow under lethal ITC concentrations after chronic exposure. These data suggest the development of genetic-based resistance similar to that reported for synthetic fungicides. We also found that upon elimination of the ITCs, the fungi quickly loose the ability to grow in the presence of lethal ITCs concentrations. This suggests that the fungal adaptation to ITCs exposure may be due to mechanisms other than those reported for synthetic fungicides in which the resistant phenotypes are generally hereditable.

The study of fungal genome responses to the stress imposed by ITCs may be a good model to study novel mechanisms used by fungi to adapt and grow in the presence of toxic compounds. In previous work, changes in gene regulation revealed by differential display analysis, were performed by exposing *Alternaria brassicicola* for a short time to allyl-isothiocyanate and benzyl-isothiocyanate (Sellam et al., 2006). The authors found the upregulation of a glutathione S-transferase gene, designated *AbGst1*, and demonstrate the transferase activity of the protein encoded by *AbGst1* in the presence of allyl-isothiocyanate (AITC) and benzyl-isothiocyanate (BITC) *in vitro*. In continued studies with *A. brassicicola* exposed for a short period of time to AITC, the authors found upregulation of several genes, including glutathione transferases, glutathione peroxidase, γ-glutamyl-cysteine synthetases, thioredoxins, thioredoxin synthetases and oxidoreductases (Sellam et al., 2007). Beside, the AITC treatment induced one cytochrome P450-encoding gene, 10 genes encoding membrane transporters and one gene encoding a positive-acting sulphur regulatory protein. The authors suggested that AITC treatment generates reactive oxygen species, leading to the activation of the conserved ITC-detoxification mechanism mediated by phase I and phase II enzymes.

Although the experiments described above studied molecular responses of *A. brassicicola* to ITCs, it is important to note that this specie of *Alternaria* is a specific pathogen which infects only *Brassica* spp., endowed with natural resistance to ITCs including specific de-toxification mechanisms. To our knowledge, there are no similar studies reporting the effect of ITCs on non-host specific and opportunistic fungi such as *A. alternata*, *A. tenuissima*, or related saprobic species. Moreover, there have been no studies examining genome-wide changes in response to the toxic effects of ITCs using polymorphic DNA markers as opposed to expression analysis of specific genes. However, a number of techniques have been developed in recent years that would permit such an analysis based on polymerase chain reaction (PCR), including random amplified polymorphic DNA (Williams et al., 1990) and amplified fragment length polymorphisms (Vos et al., 1995). Other PCR-based techniques include analysis of simple sequence repeats (SSRs) or microsatellites (Wu and Tanksley, 1993), and inter simple sequence repeats (ISSRs) (Zietkiewicz et al., 1994).

Microsatellites or SSR are composed of tandemly repeated, simple DNA sequence motifs of as many as six nucleotides in length. These *loci* are commonly found in both prokaryotic and eukaryotic genomes and typically are highly polymorphic within species and populations (Dettman and Taylor, 2004). Moreover, they had been found to exist in 5´-untranslated regions, 3´-untranslated regions, exon and intron sections of genes (Lawson and

Zhang, 2006). SSRs have been used as markers associated with phenotypic traits like 2-propenil glucosinolate content in *Brassica juncea* (Ripley and Roslinsky, 2005), disease resistance genes in a segregating population of a cross between *Cicer arietinum* and *Cicer reticulatum* (Ratnaparkhe et al., 1998), or different types of stress such as salt tolerance in *Oryza sativa* (Kaushik et al., 2003). The polymorphisms of SSRs are derived mainly from variability in length, due to changes in the number of repeats units, rather than in the primary sequence (Ellegren, 2004). Length instability appears to be a product of the increased susceptibility of repetitive DNA to slipped strand mispairing during DNA replication (Leonard et al., 2003). Furthermore, these phenomena may be increased in genomes facing the pressure of either abiotic or biotic stresses. Indeed, in a study to examine mutations in hexaploid wheat following exposure to the wheat pathogen *Fusarium graminearum*, Schmidt and Mitter (2004) found mutations in microsatellite *loci* previously mapped to chromosomes carrying a putative *Fusarium* head blight (FHB) resistance gene which were induced by FHB infection. In addition, trees of *Pinus sylvestris* growing nearby Chernobyl and exposed to chronic ionizing irradiation showed alterations in microsatellite *loci* as well (Vornam et al., 2004). Studies on bacteria have shown that unstable, mutation-prone microsatellite *loci* can be used to maintain the high level of phenotypic diversity required for successful exploitation of variable environments (van Belkum, 1999).

Even though SSRs show high levels of polymorphism which make them effective markers for genome-wide analysis, the time and cost of finding SSR motifs as well as the design of primers against flanking SSR regions have restricted their widespread use. In contrast, inter simple sequence repeat (ISSR) markers are easier to develop because prior knowledge of the target sequences flanking the SSR regions is not required. These markers use primers designed to anneal against microsatellite sequences and have a dominant inheritance. Most commonly, they have been used for phylogenetic studies in insects (Hundsdoerfer and Wink, 2006), plants (Mary et al., 2006; Levi et al., 2005; Vijayan et al., 2004), and fungi (Menzies et al., 2003; Hong et al., 2006). However, ISSR analysis has not been used to analyze the alterations in the genome as a consequence of abiotic or biotic stressors.

Because ISSR primers target the SSR regions, changes in the microsatellites size can eliminate or create ISSR primer sites which in turn can induce polymorphism in the ISSR regions. Thus, ISSR markers can be used to indirectly study the changes in SSR region sizes (Leroy et al., 2000). Because of the above mentioned, the objective of the present study was to use ISSR analysis to examine simple sequence repeat polymorphism in *Alternaria* strains isolated from tomato and cabbage and to compare native polymorphisms with that exhibited in strains adapted to lethal levels of allyl-isothiocyanate and benzyl-isothiocyanate.

MATERIALS AND METHODS

Alternaria cultures

Strains of *Alternaria* were isolated from either tomato fruit or cabbage collected in a commercial store located at Hermosillo, México. The isolates were cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit), and the colony and sporulation characteristics were confirmed according to Pryor and Michailides (2002) and Simmons (1995).

ITC adaptation of strains

Monospore cultures of *Alternaria* from tomato and cabbage adapted or tolerant to isothiocyanates were created according to Orth et al. (1994). The isolates were independently exposed to gradually increased concentrations of AITC and BITC, following the filter paper protocol reported by Troncoso-Rojas et al. (2005). Briefly, at the beginning of the experiment, each isolate was exposed to low concentrations of ITCs (0.01 mg/mL), with increments of 0.01 up to 0.08 mg/mL of AITC and up to 0.6 mg/mL of BITC. The exposure time to each concentration of ITC was maintained until the isolate was able to grow under the selective pressure. During this time, each original wild-type strain was maintained under the same conditions but without ITC. Thus, from every isolated wild-type strain, a resistant strain was developed in such a way that the wild-type strain was an isogenic line with the resistant strains differing only by the changes induced by the ITC over the genome.

Each strain of *Alternaria* was considered adapted or tolerant to the ITC toxic effect when it was able to grow at an isothiocyanate level in which the original wild-type strain did not grow. Some of the strains failed to keep growing during adaptation process and were discarded from the experiment. By the end of the adaptation process, ten wild-type strains, five from each host, and their adapted derivative strains were available for the experiment. The different strains of *Alternaria* in this study were designed as follow: ATW, ATA, and ATB stand for *Alternaria*-tomato-wild-type, *Alternaria*-tomato-allyl isothiocyanate adapted strains, and *Alternaria*-tomato-benzyl isothiocyanate adapted strains, respectively. Similarly, ACW, ACA, and ACB referred to *Alternaria*-cabbage-wild-type, *Alternaria*-cabbage-allyl isothiocyanate adapted strains, and *Alternaria*-cabbage-benzyl isothiocyanate adapted strains, respectively. The number following each strain designation, 1 through 5, refers to the original wild-type strain or in the case of ITC-adapted strains, the wild-type strain from which the adapted strain was derived. Thus, there were five different *Alternaria* wild-type strains isolated from tomato fruit (ATW-1, ATW-2, ATW-3, ATW-4, and ATW-5), and their corresponding AITC and BITC adapted strains (ATA-1, ATA-2, ATA-3, ATA-4, ATA-5, ATB-1, ATB-2, ATB-3, ATB-4 and ATB-5). Similarly, from cabbage, there was five available *Alternaria* wild-type strains (ACW-1 ACW-2, ACW-3, ACW-4, ACW-5), and their corresponding AITC and BITC adapted strains (ACA-1, ACA-2, ACA-3, ACA-4, ACA-5, ACB-1, ACB-2, ACB-3, ACB-4, ACB-5).

Morphological characterization

To evaluate if any morphological changes resulted from ITC adaptation, colony and sporulation characteristics were determined for all wild-type of *Alternaria* isolated from tomato and cabbage as well as for the adapted strains to either AITC or BITC, following the

protocol reported by Pryor and Michailides (2002). Colony morphology was characterized following transfer to Petri dishes containing PDA. Dishes were incubated at 26°C in darkness for 10 days. After incubation, cultures were examined visually for colony color, colony margin, colony texture, and the development of pigments or crystals in the agar medium. Colony color was determined using Ridgway's color standards (Ridgway, 1912). Colony texture was determined using descriptions by Nobles (1948). Based upon these characteristics, isolates were placed into one of four cultural groups: A, B, C, and D, previously established in studies on *Alternaria* isolated from other crops (Pryor and Michailides, 2002; Hong et al., 2006). Cultural groups A, B, C, and D correspond to the species *A. alternata, A. tenuissima, A. arborescens,* and *A. infectoria*, respectively.

To characterize the sporulation apparatus, isolates were transferred onto plastic Petri dishes (93 x 15 mm) containing 0.5 x PDA media (Pryor and Michailides, 2002) and incubated under light without humidity control for 7 to 10 days at 26°C. After incubation, cultures were examined with a stereoscopic microscope Iroscope NZ-14T for morphological characterization of the sporulation apparatus. Isolates were grouped morphologically and placed into one of four sporulation groups: 1, 2, 3, and 4, previously established and based upon characteristics of the sporulation apparatus, including length of conidial chains, presence of elongated secondary conidiophores, and manner by which branching of conidial chains occurred (Pryor and Michailides, 2002; Hong et al., 2006). Sporulation groups 1, 2, 3 and 4 correspond to the species *A. alternata, A. tenuissima, A. arborescens,* and *A. infectoria*, respectively.

The colony and sporulation characteristics of four commonly occurring saprobic *Alternaria* species: *A. alternata*, *A. tenuissima*, *A. arborescens*, and *A. infectoria* (EGS 34-016, EGS 34-015, EGS 39-128, and EGS 27-193, respectively) were also determined and compared to those of tomato and cabbage isolates. Morphological characterization of these isolates was performed in the same manner as for the tomato and cabbage isolates.

DNA isolation

Alternaria genomic DNA from both wild-type and the corresponding isogenic strains adapted to either AITC or BITC, were obtained from fungi mycelia growing in Petri-dish containing PDA, according to the protocol reported by Lecellier and Silar (1994). Briefly, a small piece of each isolate mycelia was inoculated on PDA agar covered with sterilized cellophane paper (Cassago et al., 2002), and incubated at 26°C in darkness for 10 days, until mycelial tips reached the margin of the agar plate. Mycelium was harvested, freeze-dried and ground with a sterile toothpick in a 1.5 mL Eppendorf tube. Lysis buffer was added (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 100 mM NaCl, 2% SDS, 2% PVP (w/v) and 1/1000 volume of RNase enzyme) and the reaction was incubated for 30 min at 75°C. After this time, 1 volume of Tris-HCl equilibrated phenol was added to each tube, vortex during for 1 min and centrifuged at 12000 rpm during 10 min in a microfuge. The aqueous phase was transferred to a new tube and a second extraction was performed with 1 volume of phenol:chloroform (1:1). The aqueous phase was transferred to a new tube and mixed with 1 volume of chloroform and centrifuged. The aqueous phase was transferred to a new tube and the DNA was precipitated by the addition of 1 volume of chilled isopropyl alcohol. The nucleic acid pellet resulting from the centrifugation was washed with 70% ethanol and vacuum-dried. The nucleic acids were re-suspended in 50 µL of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). DNA was resolved in 1% agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA disodium salt) and visualized by UV illumination after staining with ethidium bromide. The DNA concentration in each preparation was determined with a spectrophotometer UV-VIS Cary 50 Bio (Varian, Inc. Co. Palo Alto, CA, USA), and it was adjusted to a final concentration of 10 ng/µL with TE buffer.

Inter simple sequence repeat (ISSR) amplification

Five primers were tested to anneal with several microsatellite *loci* located in the *Alternaria* genome; $(GACA)_4$: 5'-GACAGACAGACAGACA-3'; AAG_8:5'-AAGAAGAAGAAGAAGAAGAAGAAG-3'; ACA_5: 5'-ACAACAACAACAACA-3'; T3B: 5'-AGGTCGCGGGTTCGAATCC-3' and M13: 5'-GAGGGTGGCGGCGGTTCT-3'. PCR amplification was performed using a GeneAmp PCR Mastercycler (System 9700, PE Biosystems, USA) in a total volume of 50 µL containing 10 ng/µL DNA, 0.5 µM of each primer, 0.25 mM of dNTP, 2.5 mM $MgCl_2$, and 5.0 Units of Taq DNA polymerase in 1X PCR buffer (Promega Corporation, Madison WI). PCR conditions were: 94°C for 10 min, followed by 40 cycles of 1 min at 94°C, 1:30 min at 50°C, and 2 min at 72°C. Negative controls were included. PCR products were separated by gel electrophoresis on 1% (w/v) agarose in TBE buffer and photographed after staining with ethidium bromide.

ISSR analysis

For each primer, resulting polymorphic bands from each isolate were scored for the presence or absence of a given fragment and a binary matrix was constructed. Cluster analysis of the data matrix was performed by the Unweighted Pair Group Method with Arithmetic Means (UPGMA) with Jaccard's similarity coefficient (Sneath and Sokal, 1973) and the goodness of fit was measured by cophenetic correlation (r) analysis using the software NTSYSpc ver. 2.1 (Exeter Software, Setauket, NY).

RESULTS

Morphological characterization of *Alternaria* strains wild-type adapted to ITCs

All wild-type isolates of *Alternaria* recovered from both tomato tissue (ATW) and cabbage tissue (ACW), produced colonies more than 80 mm in diameter after 12 days of incubation and most of them showed olive green to dark olive green color. One colony isolated from tomato (Table 1) and three colonies isolated from cabbage (Table 2) produced white-colored crystals in the agar medium underneath the mycelial mat suggesting the production of diffusible secondary metabolites.

The sporulation apparatus of wild-type isolates included conidia that were typically of yellowish-brown color, ovate in shape with the presence of transverse and longitudinal septa, formation of conidial chains 6-14 in length and the development of numerous secondary, and occasionally tertiary, chains 2-8 conidia in length. Chain branching occurred in a sympodial manner through the elongation of basal conidiophores to existing spores and subsequent conidium formation or through the lateral growth of secondary conidiophores from median conidial cells and subsequent conidium formation. The development of apical conidium extensions was minimal. These collective characteristics placed all isolates in the sporulation

Table 1. Morphology of the *Alternaria* strains isolated from tomato as well as the isothiocyanate (ITC) adapted strains.

Strain	Growth rate (mm)	Day	Color	Margin	Texture	Underneath characteristic	
						Color	Pigments or crystal
ATW-1	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Diffusible white pigments
ATA-1	55	10	Dark olive green	Regular	Felty to woolly	Olive green	Absence
ATB-1	60	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ATW-2	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ATA-2	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ATB-2	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ATW-3	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ATA-3	60	10	Dark olive green	Regular	Felty to woolly	Light olive green	Absence
ATB-3	65	10	Dark olive green	White margin (1 mm)	Felty to woolly	Olive green	Absence
ATW-4	80	10	Dark olive green	Wavy	Felty to woolly	Dark olive green	Absence
ATA-4	70	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ATB-4	75	10	Dark olive green	White margin (1mm)	Felty to woolly	Dark olive green	Absence
ATW-5	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ATA-5	68	10	Dark olive green	Regular	Felty to woolly	Dark olive green	White pigments
ATB-5	75	10	Dark olive green	White margin (1 mm)	Felty to woolly	Light olive green	Whitish pigments

ATW-1 is the first isolate of *Alternaria* wild type obtained from tomato, ATA1 is an *Alternaria* isogenic with ATW-1 adapted to allyl-ITC and ATB-1 is an *Alternaria* isogenic with ATW-1 adapted to benzyl-ITC.

Table 2. Morphology of the *Alternaria* strains isolated from cabbage tissue as well as the isothiocyanate (ITC) adapted strains.

Strain	Growth rate (mm)	Day	Color	Margin	Texture	Underneath characteristic	
						Color	Pigments or crystal
ACW-1	80	10	Dark olive green	Regular	Felty to woolly	Dark green brown	Whitish pigments
ACA-1	75	10	Dark olive green	Regular	Felty to woolly	Green brown	Whitish pigments
ACB-1	80	10	Dark olive green	Regular	Felty to woolly	Green brown	Whitish pigments
ACW-2	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ACA-2	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ACB-2	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ACW-3	56	10	Dark olive green	Wavy	Felty to woolly	Dark olive green	Whitish pigments
ACA-3	40	10	Dark olive green	Wavy	Felty to woolly	Olive green	Whitish pigments
ACB-3	45	10	Dark olive green	Wavy, white margin (1 mm)	Felty to woolly	Olive green	Absence
ACW-4	70	10	Dark olive green	Wavy	Felty to woolly	Olive green	Absence
ACA-4	56	10	Dark olive green	Wavy	Felty to woolly	Dark olive green	Absence
ACB-4	56	10	Dark olive green	Regular	Felty to woolly	Dark olive green	Absence
ACW-5	80	10	Dark olive green	Regular	Felty to woolly	Dark olive green	White pigments
ACA-5	80	10	Dark olive green	Regular	Felty to woolly	Olive green	White pigments
ACB-5	80	10	Dark olive green	Regular, white margin (1 mm)	Felty to woolly	Green brown	White pigments

ACW-1 is the first *Alternaria* wild type obtained from cabbage, ACA-1 is an *Alternaria* isogenic with ACW-1 adapted to allyl-ITC and ACB-1 is an *Alternaria* isogenic with ACW-1 adapted to benzyl-ITC.

group 1 and most similar to the *A. alternata* representative culture. Strains of *Alternaria* isolated from tomato and adapted to AITC (ATA) and BITC (ATB), were obtained after prolonged and repeated exposure starting from concentrations of 0.08 and 0.6 mg/mL of AITC and BITC, respectively. ATA and ATB colonies showed a much slower growth rate as compared with the wild-type strains. After 10 days of incubation at 26°C, colony growth of ATA and ATB strains was generally 50 and 60 mm in diameter, respectively; whereas ATW showed more than 80 mm by this time. However, after 12 days, no differences in colony size were recorded. No

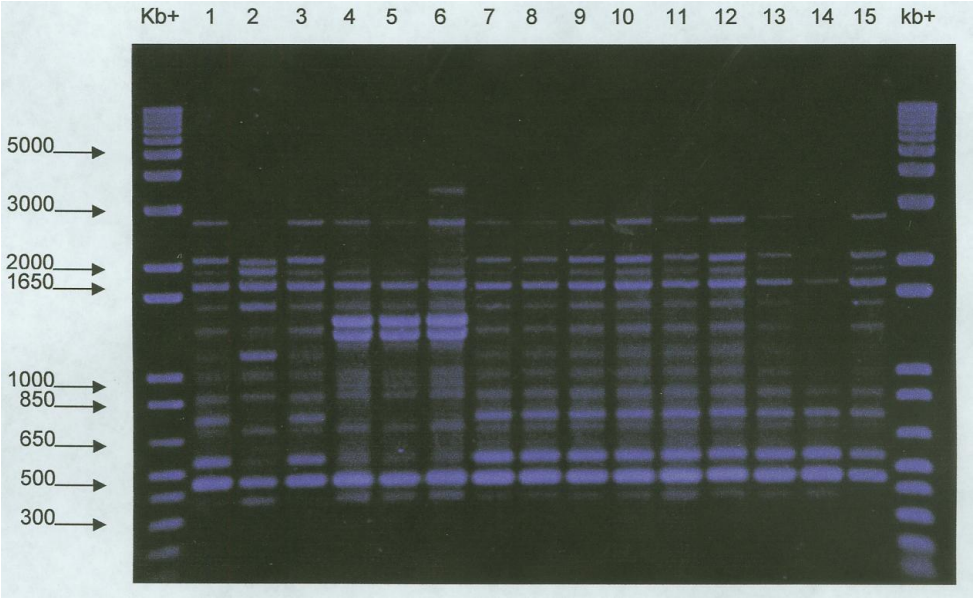

Figure 1. Polymorphism of the inter simple sequence repeats regions amplified using the primer T3B from the genomic DNA of different isolates of *Alternaria alternata* obtained from tomato tissue. It included the wild type and the corresponding isogenic lines adapted to either allyl-isothiocyanate or benzyl-isothiocyanate. DNA molecular weight marker: kb+ DNA ladder in both the first and last lanes. Line 1, ATA-1; line 2, ATB-1; line 3, ATW-1; line 4, ATA-2; line 5, ATB-2; line 6, ATW-2; line 7, ATA-3; line 8, ATB-3; line 9, ATW-3; line 10, ATA-4; line 11, ATB-4; line 12, ATW-4; line 13, ATA-5; line 14, ATB-5; line 15, ATW-5. ATW-1 is first isolate of *A. alternata* wild type obtained from tomato, ATA-1 means *A. alternata* isogenic with ATW-1 adapted to allyl-ITC, and ATB-1 is *A. alternata* isogenic with ATW-1 adapted to benzyl-ITC.

color changes were observed in any of the colonies of ATA and ATB with respect to wild-type strains (ATW). One of the ATA colonies showed an irregular edge and a dark green center in the agar medium underneath the mycelial mat, which is more similar to cultural group C. For strains adapted to BITC, all exhibited a red-brown color during the first five incubation days; also characteristic of cultural group C, but a typical dark olive green color was observed at the end of the incubation time, which is most characteristic of cultural group A.

Strains of *Alternaria* isolated from cabbage adapted to AITC (ACA) and BITC (ACB), were obtained using the same AITC and BITC concentrations mentioned above. As with the tomato isolates, cabbage strains adapted to toxic effect of either isothiocyanate.

Polymorphism in the inter simple sequence repeat of *Alternaria* strains adapted to ITCs

Out of the five microsatellites primers tested, four revealed polymorphism between wild-type and ITC-adapted *Alternaria*: T3B, $GACA_4$, ACA_5 and M13. For reasons of space, only two primers are displayed to reveal the resolution of the amplified ISSR regions by agarose gel electrophoresis (Figures 1 and 2). The analysis of the bands sizes amplified by the different AITC and BITC showed a slow growth rate as compared with the wild-type strains (ACW). After 10 days at 26°C, colony growth of ACA and ACB was 40 and 50 mm in diameter, respectively, compared with more than 80 mm for ACW strains. No color changes were observed in all strains adapted to the ITCs with respect to wild-type strains. Some ACB strains (two out of five) showed an irregular colony margin more similar to cultural group C, and revealed a red-brown pigment during the first incubation days. However, by the end of incubation, all isolates exhibited the characteristic dark olive green color more typical of cultural group A. From all the above mentioned, there was not a clear phenotype that can be ascribed to the adaptation of the *Alternaria* strains to the microsatellite primers are presented in Tables 3 and 4. Only strains showing polymorphism between the wild-type and corresponding adapted strains either to AITC or BITC are shown in the mentioned tables.

Figure 1 reveals the inter simple sequence repeats amplified using the T3B primer in five wild-type strains of *Alternaria* isolated from tomato (ATW) and the corresponding adapted strains to the presence of AITC (ATA strains) and BITC (ATB strains). For this primer, approximately 8 to 16 fragments ranging in the size from 383 to 3484 bp were amplified in the different strains. Strains 1, 3, 4, and 5 (ATW1, ATW3, ATW4, and ATW5) revealed similar fingerprinting patterns, suggesting

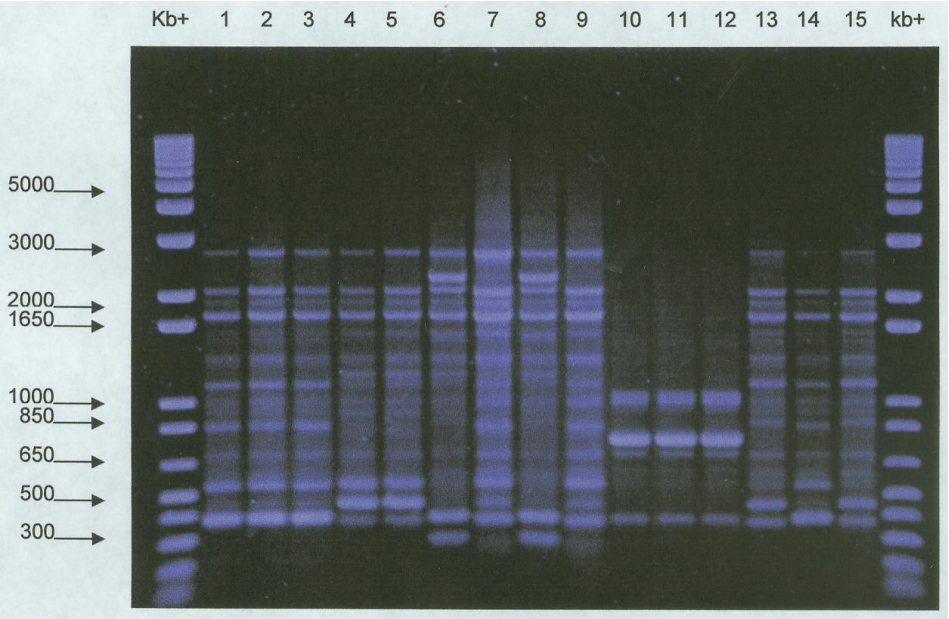

Figure 2. Polymorphism of the inter simple sequence repeats regions amplified using the primer T3B from the genomic DNA of different isolates of *Alternaria alternata* obtained from cabbage tissue. It is included the wild type and the corresponding isogenic lines adapted to either AITC or BITC. DNA molecular weight marker: kb+ DNA ladder in both the first and last lanes. Line 1, ACA-1; line 2, ACB-1; line 3, ACW-1; line 4, ACA-2; line 5, ACB-2; line 6, ACW-2; line 7, ACA-3; line 8, ACB-3; line 9, ACW-3; line 10, ACA-4; line 11, ACB-4; line 12, ACW-4; line 13, ACA-5; line 14, ACB-5; line 15, ACW-5. ACW-1 is first isolate of *A. alternata* wild type obtained from cabbage, ACA-1 means *A. alternata* isogenic with ACW-1 adapted to allyl-ITC, and ACB-1 is *A. alternata* isogenic with ACW-1 adapted to benzyl-ITC.

genetically similar fungi; whereas strain 2 (ATW-2) revealed a distinct pattern suggesting a distinct genotype. Regarding AITC-adapted strains (ATA), the ISSR fragments of 3484 and 2103 bp were not amplified in ATA-2, but were amplified in ATW-2 (Table 3). In the case of BITC-adapted strains (ATB), the ISSR fragments of 565, 773 and 2752 bp were not amplified in ATB-1, but they were amplified in both ATW-1 (wild-type strain) and in ATA-1. In contrast, the ISSR fragment with size of 383 bp was amplified in the ATB-1 strain, but it was not observed in the corresponding wild-type strain ATW-1 or in ATA-1. A number of other polymorphisms in ISSR amplified fragments were observed in experiments done with wild-type strains 2 and 5, all showing a reduction in amplified products. In summary, four polymorphisms were noted in AITC-adapted strains whereas 13 polymorphisms were noted in BITC-adapted strains compared to wild-type strains.

Figure 2 reveals the inter simple sequence repeats amplified by the T3B primer in five wild-type strains of *Alternaria* isolated from cabbage (ACW) and the corresponding adapted strains to the presence of AITC (ACA strains) and BITC (ACB strains). Strains 1, 2, 3, and 5 (ACW1, ACW2, ACW3, and ACW5) revealed similar fingerprinting patterns, suggesting genetically similar fungi, whereas strain 4 (ACW-4) revealed a distinct pattern suggesting a distinct genotype. In ACW-4 strain, the primer T3B amplified only one ISSR regions with a size of 859, which was not found present in the ACA-4 and ACB-4 strains; whereas in the others strains isolated from cabbage, T3B amplified nine ISSR regions with sizes ranging from 321 to 2314 bp (Table 4). Regarding AITC-adapted strains (ATA), the ISSR fragments of 321 and 2314 bp were not amplified in ACA-2, but were amplified in ACW-2; likewise, the ISSR fragment of 1532 bp was not amplified in ACA-1 but it was found in ACW-1 (Table 4). Regarding BITC-adapted strains (ACB strains), the ISSR fragments of 321 and 2,314 were not amplified in ACB-2; fragments of 473, 553, 628 and 859 bp were not amplified in ACB-3, the fragment 859 was not amplified in ACB-4, but those fragments were amplified in all corresponding wild-type strains. In contrast, the ISSR fragments 473 and 553 bp were amplified in ACB-2, and fragments 945, 1238, 2314 bp were amplified in ACB-3, but they were not amplified in the corresponding wild-type strains. In summary, 7 polymorphisms were noted in AITC-adapted strains whereas 12 polymorphisms were noted in BITC-adapted strains.

Amplification of the ISSR regions using the primer $GACA_4$ revealed numerous polymorphisms in *Alternaria* strains adapted to either AITC or BITC. In summary,

Table 3. Inter simple sequence repeats regions amplified by different primers from the genomic DNA of *Alternaria* strains isolated from tomato.

Primer T3B								
ATA-1*	**ATB-1**	**ATW-1**	**ATA-2**	**ATB-2**	**ATW-2**	**ATA-5**	**ATB-5**	**ATW-5**
			---	---	3484			
2752	---	2752	---	---	---	2752	---	2752
			---	---	2103	2103	---	2103
						1929	---	1929
						1561	---	1561
						1380	---	1380
						1010	---	1010
773	---	773						
565	---	565						
---	383	---				383	383	---

Primer $GACA_4$								
ATA-1	**ATB-1**	**ATW-1**	**ATA-2**	**ATB-2**	**ATW-2**	**ATA-3**	**ATB-3**	**ATW-3**
			---	2814	2814			
			2471	2471	---	2471	---	2471
			2232	2232	---			
1647	---	1647				1647	---	1647
						---	---	1169
1012	---	1012						
---	843	---						
812	812	---						

Primer ACA_5		
ATA-1	**ATB-1**	**ATW-1**
---	2275	---
---	1223	---
---	1180	---
1065	---	1065
587	---	587

Primer M13					
ATA-1	**ATB-1**	**ATW-1**	**ATA-4**	**ATB-4**	**ATW-4**
---	---	1509	1509	---	---
---	---	1422	1422	---	---
			1158	1158	---
1044	---	1044	1044	1044	---
			963	963	---
878	---	878	878	878	---
			761	761	---
			698	698	---
			558	558	---
			464	464	---
---	---	393	393	393	---

It included the wild type and the isogenic lines adapted to either allyl-isothiocyanate or benzyl-isothiocyanate. For reasons of clarity, *Alternaria*strains in which the isothiocyanate treatment did not induce polymorphism are not included. Also, common bands are not included either. ATW-1 is first isolate of *Alternaria* wild type obtained from tomato, ATA1 is an *Alternaria* isogenic with ATW-1 adapted to allyl-ITC and ATB-1 is an *Alternaria* isogenic with ATW-1 adapted to benzyl-ITC. A dotted line indicates that the corresponding band is not present.

Table 4. Inter simple sequence repeats regions amplified by different primers from the genomic DNA of *Alternaria* strains isolated from cabbage.

Primer T3B														
ACA-1*	**ACB-1**	**ACW-1**	**ACA-2**	**ACB-2**	**ACW-2**	**ACA-3**	**ACB-3**	**ACW-3**	**ACA-4**	**ACB-4**	**ACW-4**			
			---	---	2314	---	2314	---						
---	1532	1532												
						1238	1238	---						
					---	---	945	---						
						859	---	859	---	---	859			
						628	---	628						
			553	553	---	553	---	553						
			473	473	---	473	---	473						
			---	---	321									
Primer $GACA_4$														
ACA-1	**ACB-1**	**ACW-1**	**ACA-2**	**ACB-2**	**ACW-2**	**ACA-3**	**ACB-3**	**ACW-3**				**ACA-5**	**ACB-5**	**ACW-5**
												3317	---	3317
---	2589	2589												
			---	---	2248	---	2248	---				2248	---	2248
			---	---	1995	---	1995	---						
			1886	1886	---	1886	---	1886						
			1230	1230	---									
---	1071	---	1071	1071	---									
			822	822	---	822	---	822						
						597	597	---						
			553	553	---									
						---	531	531						
						---	413	413						
Primer ACA_5														
ACA-1	**ACB-1**	**ACW-1**	**ACA-2**	**ACB-2**	**ACW-2**	**ACA-3**	**ACB-3**	**ACW-3**	**ACA-4**	**ACB-4**	**ACW-4**	**ACA-5**	**ACB-5**	**ACW-5**
			---	---	2994							2994	---	2994
												2812	---	2812
									---	1919	---			
			---	---	1778				---	1778	---	1778	---	1778
						1541	---	1541						
									---	1333	---			
			---	---	1242									
									1079	---	1079			

Table 4. Contd.

												903	---	903
550	---	550	550	550	---	550	---	550	---	550	---			
						516	---	516						
318	---	318	318	318	---									
Primer M13														
ACA-1	**ACB-1**	**ACW-1**	**ACA-2**	**ACB-2**	**ACW-2**	**ACA-3**	**ACB-3**	**ACW-3**	**ACA-4**	**ACB-4**	**ACW-4**	**ACA-5**	**ACB-5**	**ACW-5**
						1517	---	1517						
---	1410	---	---	1410	1410							1410	1410	---
						1179	---	1179						
---	1117	---	---	---	1117	---	1117	---	---	---	1117	1117	1117	---
---	1072	---	---	1072	---	1072	---	1072						
---	974	---	---	974	974							974	974	---
---	881	---	---	881	---	881	---	881	---	881	---			
			---	---	810	---	810	---						
---	766	---				766	---	766	766	---	766	766	766	---
---	567	---	---	567	567							567	567	---
---	475	---	---	475	475							---	475	---
---	392	---	---	---	392							---	392	---

It included the wild type and the isogenic lines adapted to either allyl-isothiocyanate or benzyl-isothiocyanate. For reasons of clarity, *Alternaria* strains in which the isothiocyanate treatment did not induce polymorphism are not included.Also, common bands are not included, either. ACW-1 is the isolate of *Alternaria* wild type obtained from cabbage, ACA-1 is an *Alternaria* isogenic with ACW-1 adapted to allyl-ITC and ACB-1 is an *Alternaria* isogenic with ACW-1 adapted to benzyl-ITC. A dotted line indicates that corresponding band is not present.

three polymorphisms were noted in AITC-adapted tomato isolates; whereas nine polymorphisms were noted in BITC-adapted tomato strains (Table 3). In addition, 14 polymorphisms were noted in AITC-adapted cabbage strains; whereas 15 polymorphisms were noted in BITC-adapted cabbage strains (Table 4).

Summarizing results from primer ACA_5 shows that no polymorphisms were noted between AITC-adapted tomato strains, whereas five polymerphisms were noted in BITC-adapted tomato strains (Table 3). Similarly, five polymorphisms were noted in AITC-adapted cabbage strains whereas 19 polymorphisms were noted in BITC-adapted strains (Table 4). In the case of the primer M13, five polymorphisms were noted in AITC-adapted tomato strains whereas 14 polymorphisms were noted in BITC-adapted strains (Table 3). Beside, 13 polymorphisms were noted in AITC-adapted cabbage strains whereas 31 polymorphisms were noted in the BITC-adapted cabbage strains (Table 4).

Overall, when combining data from all four primers, 51 polymorphisms were noted in AITC-adapted strains whereas 118 polymorphisms were noted in AITC-adapted strains whereas 118 polymorphisms were noted in the BITC-adapted strains across both hosts. Looking at the data by host, 53 polymorphisms were noted for tomato strains whereas 116 polymorphisms were noted for cabbage strains across both ITC's. In terms of primer productivity, 36, 41, 29, and 63 polymorphisms were revealed for combined AITC/BITC-adapted strains and tomato/cabbage strains for primers T3B, $GACA_4$, ACA_5, and M13, respectively.

Cluster analysis of the ISSR regions amplified in the wild-type *Alternaria* strains isolated from tomato (ATW) compared with those amplified from corresponding AITC (ATA) and BITC (ATB) adapted strains revealed variation among the wild-type strains (Figure 3). The lowest coefficient of similarity among wild-types was 61% and the highest was 93.5%. Following exposure to the isothiocyanates, the resulting polymorphisms

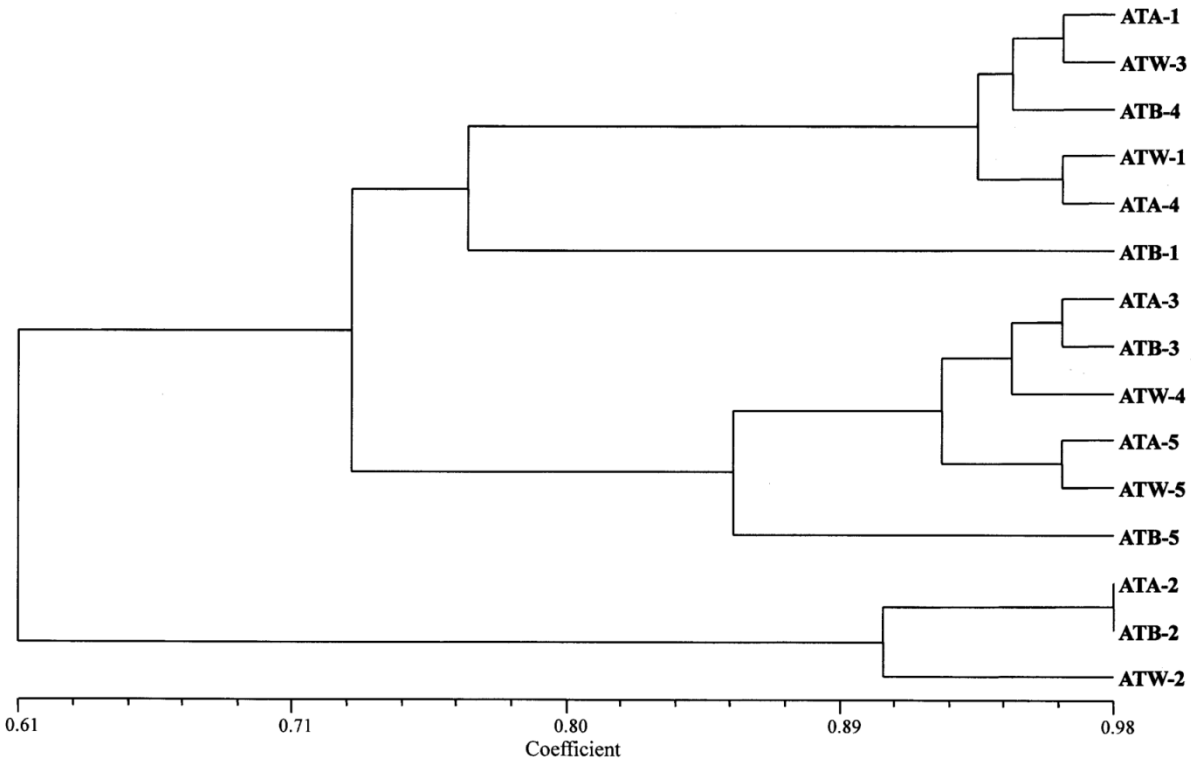

Figure 3. Cluster analysis of the ISSR regions amplified in the *Alternaria* strains isolated from tomato (ATW´s) as well as the ISSR regions of the corresponding allyl-isothiocyanate (ATA´s) and benzyl-isothiocyanate (ATB's) adapted strains. Meaning of symbols: ATW-1 is first isolate of *A. alternata* wild type obtained from tomato, ATA-1 means *A. alternata* isogenic with ATW-1 adapted to allyl-ITC, and ATB-1 is *A. alternata* isogenic with ATW-1 adapted to benzyl-ITC).

did not reveal a clear pattern. Moreover, the exposure of strains to any of the ITC's did not induce large polymorphisms; although this was not always the case. For example, exposure of ATW-1 strain created the strains ATA-1 and ATB-1, each with different polymorphisms. Similarity coefficient between isolates was 76.3%. Further, each one showed a different coefficient of relatedness as compared with the original ATW-1 with the similarity coefficient between ATA-1 and ATW-1 of 93.1% and between ATB-1 and ATW-1 of 72.4%. In contrast, in the case of the ATW-2, resulting polymorphisms following exposure were minor and the similarity coefficient between the derived strains and the wild- type was 91.5% for both ATA-2 and ATB-2. Cluster analysis of the ISSR regions amplified in the *Alternaria* strains isolated from cabbage (ACW) compared with those of the corresponding AITC (ACA) and BITC (ACB) adapted strains revealed a similar pattern as that revealed from analysis of the tomato isolates (Figure 4). In a similar manner as the strains isolated from tomato, the derived strains following exposure showed different polymorphisms from the wild-type in such a way that most of them fall into different clades of the dendogram from the wild-type. Similarity coefficient among wild-type strains isolated from cabbage was 53 to 73.6%, compared with 61-95% for wild-type strains isolated from tomato, suggesting an initial higher exposure showed different polymorphisms from the wild-type in such a way that most of them fall into different clades of the dendogram from the wild-type. Similarity coefficient among wild-type strains isolated from cabbage was 53 to 73.6%, compared with 61-95% for wild-type strains isolated from tomato, suggesting an initial higher variability in the strains isolated from cabbage. In four out of five strains adapted to the allyl-isothiocyanate (ACW-1, ACW-3, ACW-4 and ACW-5), the adapted strains showed a rather similar ISSR pattern compared with the corresponding ACW strains, and similarity coefficients varied from 92.3% for ACW-5/ACA-5 to 98.4% for ACW-1/ACA-1. However, for the case of the ACW-2, the coefficient of similarity with ACA-2 was of 66.1%. In contrast, the coefficients related with the changes in ISSR polymorphisms of ACW-3 and ACW-4 strains after BITC exposure was much greater and varied from 63.07% for ACB-3 to 86.1% for ACB-4. These data clearly reveal a higher variability in polymorphism by the BITC exposure as compared with the AITC treatment, which agrees with the higher polymorphisms recorded in

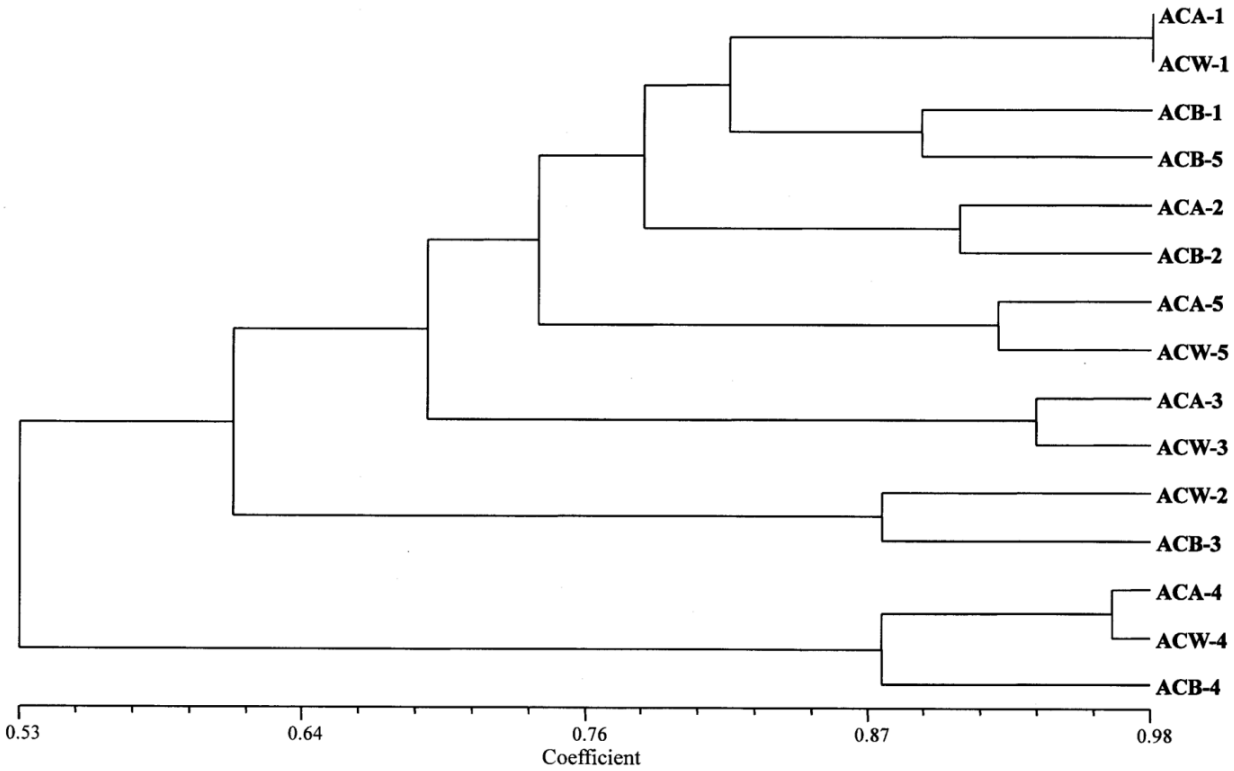

Figure 4. Cluster analysis of the ISSR regions amplified in the *Alternaria* strains isolated from cabbage (ACW´s) as well as the ISSR regions of the corresponding allyl-isothiocyanate (ACA´s) and benzyl-isothiocyanate (ACB's) adapted strains. ACW-1 is first isolate of *A. alternata* wild type obtained from cabbage, ACA-1 means *A. alternata* isogenic with ACW-1 adapted to allyl-ITC, and ACB-1 is *A. alternata* isogenic with ACW-1 adapted to benzyl-ITC.

BITC strains as compared with AITC strains.

DISCUSSION

Morphological examination of *Alternaria* strains isolated from tomato and cabbage tissue were consistent with the characteristics of the *A. alternata* morphological group. Although robust morphological delimitation of *A. alternata* from closely related sister taxa such as *A. tenuissima* or *A. arborescens* is often difficult (Weir et al., 1998; Hong et al., 2005), the characters exhibited by the tomato and cabbage strains permits the placement of these taxa as *A. alternata sensu lato*. Perhaps more importantly, adaptation to either AITC or BITC did not have a significant impact on the morphology of these fungi. This is in contrast to the noted morphological plasticity exhibited by these fungi in response to other environmental changes such as light, temperature, and humidity (Simmons, 1995). Although minor cultural changes were noted among isolates after chronic exposure to lethal concentrations of allyl-isothiocyanate and benzyl-isothiocyanate, such as a slow growth phenotype as compared with the wild-type, there were no significant alterations in the primary cultural and sporulation characteristics. As a result, no change in morphological grouping took place. These results are similar with those reported by Katan et al. (1983), who found that the fungus *Venturia inaequalis*, grew slower in 50 g/mL as compared with 5 g/mL of the fungicide benomyl, but otherwise no other significant morphological or cultural changes were recorded. In contrast, morphological anomalies in some fungi were recorded with the use of an analog of sampangine, 4-bromosampangine, including excessive branching of germ tubes in *Colletotrichum fragariae* and splaying and branching of germ tubes in *Botrytis cinerea* (Abril et al., 2008).

Differences in ISSR polymorphism were observed in strains isolated from either tomato or cabbage and their corresponding strains independently treated with AITC or BITC. Analysis of ISSR polymorphism showed a higher variability for both wild-type strains and ITC adapted strains isolated from cabbage as compared with the strains isolated from tomato. Indeed, for wild-type strains isolated from cabbage, it was calculated a coefficient ranging between 49.2 and 72.3%; whereas for tomato, the range was between 65.5 and 93.1%. Also, it was recorded that 116 polymorphisms were induced by the isothiocyanates in cabbage and 53 in tomato. These results clearly show the presence in cabbage of fungi strains less genetically similar and a larger effect on the

polymorphism of those strains by the isothiocyanate treatment. Taking into account the effects on the ISSR polymorphism generated in this work, most likely this phenomena is due to the fact that the *Alternaria* strains isolated from cabbage were already under isothiocyanate exposure due to the natural presence of those chemicals in the vegetable.

In general, it was recorded a lower variation in the polymorphism by the AITC treatment as compared with the BITC treatment for both strains isolated from cabbage and tomato. Indeed, 51 polymorphisms were recorded to be associated to the AITC treatment, whereas 118 were found associated with the BITC treatment. This finding can be ascribed to the differences in the chemical properties of the functional group present in ally-isothiocyanate (2-propenyl) and benzyl-isothiocyanate (benzyl) allowing for a particular mode of action. Indeed, it had been found in experiments with human pancreatic cells, that benzyl isothiocyanate can inhibit cancer cell proliferation by direct DNA damage (Zhang et al., 2006); whereas allyl-isothiocyanate cause the same effect by a different mechanism (Xiao et al., 2003). Based on this data, it can be suggested that BITC can cause a more direct effect on the *Alternaria* DNA which can explain the presence of a larger number of polymorphisms.

The cluster analyses revealed that independently exposed *Alternata* wild-type strains showed different polymorphism pattern in response to the exposure to either AITC or BITC, which suggest that exposure to ITCs induced random mutations in the *Alternata* genome. For instance, it was recorded that strains ATW-1 and ATW-3 had a similarity of 76.9 and 96.2%, before and after exposure of the ATW-1 strain to the AITC treatment, respectively. ACW-2 and ACW-3 showed a similarity of 61.3% and after BITC exposure, ACW-2 and ACB-3 (resultant of ACW-3 exposure to BITC) had a coefficient of 87.8%. To our knowledge, no experiments had been done testing the effect of either abiotic or biotic stress on ISSR polymorphism. However, we would like to suggest that changes in ISSR patterns may be the result of changes in the corresponding flanking microsatellite regions size. Efforts are underway in our lab to experimentally test it.

In our study, the amplification of ISSR region technique was useful to find differences between wild-type strains and strains adapted to either AITC or BITC. This technique was able to find differences among the wild-type strains isolated from tomato and those isolated from cabbage. Although the characteristics of the colony morphology and conidia were similar among the wild-type strains isolated from tomato or from cabbage, differences in the ISSR polymorphism pattern were observed among those strains revealing a basal degree of variation. This variation could be due to different ancestry among strains or to different environmental stresses and selection under which the different strains were exposed to sometime before being isolated. However, more studies are needed to support this statement.

In the present study, we observed changes in the ISSR regions in the strains adapted to AITC or BITC as compared with unexposed or wild-type strains. Schmidt and Mitter (2004), tested the hypothesis that microsatellite mutation can be induced by exposure to specific external cues. The authors found that specific environmental cues induce mutations at specific microsatellite *loci*. Their results reveal that exposure to external cues (for instance, exposure to the *Fusarium* head blight) can be related to specific phenotypic traits leading to changes at microsatellite *loci* linked to those traits.

The experiments reported in this study are the first efforts using the ISSR region amplification technique to evaluate the genomic DNA changes in fungi adapted to toxic chemical compounds. The polymorphism of the different ISSR regions observed between wild-type strains and the strains adapted to AITC or BITC suggest that the microsatellites in genomic DNA of *Alternaria* were modified as an adaptative response to the ITC toxic effect. It had been shown that the alterations or modifications at microsatellite *loci* could be associated with alteration of reading frames, or modifications in activity levels of promoters and gene activation/silencing. These statements had been proven in bacteria (Bayliss et al., 2001; De Bolle et al., 2000; Rocha et al., 2002) and in higher plants (Leonard et al., 2003; Shaporova, 2008); however, further studies are needed to evaluate if the same mechanisms are active in fungi.

The effects of ITC on various macromolecules have been documented. Experiments *in vitro* have shown that allyl-isothiocyanate can react and form covalent bonds with the disulfide bonds of the oxidized glutathione (Kawakishi and Kaneko, 1987), as well as with free amino and sulfhydryl group of amino acids (Cejpek et al., 2000). These studies suggest that the ITCs can chemically react with almost any proteins through the free amino and sulfhydryl group present in the side groups of amino acids residues like lysine, arginine and cystein. In support of this hypothesis, benzyl-isothiocyanate was reacted with total white egg proteins (Kroll et al., 1993; Kroll et al., 1994a), proteic fractions from white egg (Kroll et al., 1994b), bovine sarcoplasmic proteins (Rawel and Kroll, 1995) and myoglobin (Rawel et al., 1998). In all cases, alterations in the protein isoelectric point were found as well as a reduction in the free sulfhydryl and amino group located in R groups of the amino acids were recorded.

Based upon these data, the ITC activity over the fungi appears to be rather random than specific. This may explain why a mutation which could render the fungi resistant to this chemical compound would be highly unlikely. However, differential display experiments had shown the induction of several genes upon exposure of *A. brassicicola* to the ITC (Sellam et al., 2007) which can explain the ability of the fungi to adapt and grow under

ITC selection pressure as observed in the present experiment. Experiments by suppression substractive hybridization approach are currently underway in our lab to find whether the changes in ISSR that we observed in the adapted *Alternaria* strains are modifying the regulation of specific genes.

Conclusion

Isothiocyanate exposure induced random mutations in different ISSR regions of the *Alternaria* genome which does not lead to the development of strains with a hereditable resistant phenotype. In addition, changes in the ISSR regions are perhaps a common response of the *Alternaria*'s genome to the ITCs toxic effect as a way to alter the genome regulation to make possible fungi adaptation.

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