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Mitotic crossing-over induced by two commercial herbicides in diploid strains of the fungus *Aspergillus nidulans*

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ABSTRACT. Some herbicides are suspected of promoting teratogenic, carcinogenic and mutagenic events. Detection of induced mitotic crossing-over has proven to be an indirect way of testing the carcinogenic properties of suspicious substances, because mitotic crossing-over is involved in the multistep process of carcinogenesis. We examined mitotic crossing-over induced by two commercial herbicides (diuron and trifluralin) in diploid strains of *Aspergillus nidulans* based on the homozygotization index. Low doses (2.5 µg/mL) of diuron were sufficient to increase the mean homozygotization index in 2.1 and 11.3 times for UT448// UT196 and *Dp* II-I//UT196, respectively, whereas the same dose of trifluralin increased this mean only 1.2 (UT448//UT196) and 3.5 (*Dp* II-I// UT196) times, respectively. The lower homozygotization index value found for trifluralin could be due to its interference with mitotic crossing-over in eukaryotic cells. We concluded that the diploid *Dp* II-I//UT196 of *A. nidulans* is more sensitive to organic compounds than UT448//UT196;

these compounds cause recombinational events at a greater frequency in the latter diploid. This system holds promise as an initial test for carcinogenicity of organic compounds, including herbicides.

Key words: Diuron; Trifluralin; Herbicides; Mitotic crossing-over; *Aspergillus nidulans*; Genotoxic agents

INTRODUCTION

Currently, due to an increase in the population worldwide, there has been an urge for increasing the productivity of crops and agricultural by-products. This yield increment has been obtained mainly by a massive use of pesticides to control the impacts of noxious insects, phytopathogens and weeds in agriculture. Pesticides are a multimillion dollar market corresponding to an estimated value of 25.6 million dollars per year, and herbicides constitute 45% of this amount (Dich et al., 1997). However, a series of deleterious effects on environment safety and human health have become apparent, where teratogenic, carcinogenic, and mutagenic effects have received special attention (Giacomazzi and Cochet, 2004; Nguyen-Ngoc et al., 2009). Chemical pollutants present genetic hazards to human beings of sufficient importance to warrant intensive cytogenetic study (Sousa et al., 2009). Because of these undesirable side effects of pesticides, there has been an increase in consumer awareness to avoid the use of these compounds (Zucchi et al., 2008) and/or public pressure to enhance their regulation for applications in pre- or post-harvest.

Among the pesticides used, herbicides play a crucial role in agricultural fields to avoid crop competition by weeds. Diuron [1-(3, 4-dichlorophenyl)-3, 3-dimethylurea] is a pre-emergence urea herbicide (Van Boven et al., 1990) commonly used for the control of annual and perennial grasses in a number of crops (Hardaway and Yalkowsky, 1991). It is poorly soluble in water, which leads to its accumulation in animal tissues, and therefore, diuron toxicity has been well established (Singh and Bingley, 1990). The oral LD_{50} value of diuron for rats is over 3 g/kg (Dikshith, 1990), and it has been considered moderately toxic (Hardaway and Yalkowsky, 1991). Also, it has been usually classified as highly persistent in environmental conditions, and therefore, diuron can be found in soil, sediments and water (Field et al., 2003). Trifluralin [2,6-dinitro-N, N-dipropyl-4-(trifluoromethyl) benzenamine] is a pre-emergence soil-incorporated herbicide and one of the most widely used herbicides in weed control (Deuber, 1992). Even though it has been proven to be slightly toxic to mammals (Garriott et al., 1991), several reports have demonstrated that trifluralin has genotoxic properties (Könen and Çavas, 2008; Fernandes et al., 2009). Although many reports have highlighted the genotoxic properties of both herbicides by several biosafety assays, to the best of our knowledge, none of them evaluated the potential of diuron and trifluralin to provoke recombinational alterations by homozygotization of *Aspergillus nidulans*.

The homozygotization index (HI) (Pires and Zucchi, 1994) is based on two or more *A. nidulans* diploid strains heterozygous for several well-mapped genetic markers. The homozygotization of these heterozygous markers has proven to be sensitive enough to detect recombinational alterations induced by noxious agents (Zucchi et al., 2005). Mitotic recombination is a natural tool for DNA repair, which plays an intricate association with loss of heterozygosity (Young et al., 2006), and therefore, it was demonstrated to be linked to carcinogenesis as a prior step in this process (Gupta et al., 1997). Hence, it is a highly desirable focus in efforts to

enhance our knowledge of the recombinogenic potential of suspicious compounds. Thus, the main aim of this study was to evaluate the recombinogenic effect of diuron and trifluralin on the homozygotization of genes of *A. nidulans* diploid strains.

MATERIAL AND METHODS

Aspergillus nidulans strains

The haploid strains used were from Utrecht stocks (UT448 and UT196) or obtained in our laboratory, namely *Dp* II-I, which is a *uvs* mutant obtained from N-methyl-N'-nitroN-nitrosoguanidine treatment of the UT448 strain (Zucchi, 1990; Prado and Zucchi, 1991a,b; Castro-Prado and Zucchi, 1992). Their genotypes are given in Table 1.

Table 1. Genotypes and linkage group of *Aspergillus nidulans* strains.

Strain	Genotype
UT448	*ribo*A1 (I), *paba*A124 (I), *bi*A1 (I), *Acr*A1 (II), *w*A2 (II)
UT196	*y*A2 (I), *meth*A17 (II), *pyro*A4 (IV)
Dp II-I	*w*A2 (II), *ribo*A1 (I), *paba*A124 (I), *Dp* II-I (I), *bi*A1 (I), *uvs*A (I), *Acr*A1 (II)

*ribo*A1 = riboflavin; *paba*A124 = *p*-aminobenzoic acid; *bi*A1 = biotin; *meth*A17 = methionine; *pyro*A4 = pyridoxine; Conidia color: *w*A2 = white; *y*A2 = yellow; *Acr*A1 = resistance to acriflavine; *uvs*A = sensitivity to UV; *Dp* II-I = duplication of a segment of chromosome II transposed to chromosome I, inserted into *paba-y* interval.

The diploid strains UT448//UT196, *Dp* II-I//UT196 and UT501//UT196 were prepared according to Roper's method (1952). The mutant alleles were allocated to their linkage group by mitotic haploidization (Forbes, 1959) facilitated by treatment with *p*-fluorophenylalanine (*p*FA) (Lhoas, 1961; Morpurgo, 1961).

Culture media

Complete medium (CM) and minimal medium (MM) were prepared as described by Van de Vate and Jansen (1978). Selective medium (SM) was MM supplemented with requirements needed by the particular strains. Solid medium contained 1.5% agar. Incubation was at 37°C.

Solutions

The herbicide solutions contained 2 mg/mL of the active ingredient in distilled water. Diuron was obtained from Hoechst and trifluralin from Elanco. The haploidizing agent *p*FA was diluted in 0.1 N NaOH (20 mg/mL).

Procedures

The herbicide solutions were individually and directly added to solid MM at 45°C at different final concentrations (0, 0.25, 2.5, 25.0, and 250.0 µg/mL). Conidia from each of the diploid strains were added to the center of these plates. After incubation for 5-7 days at 37°C, it was

possible to determine green sectors growing in colonies of treated diploids, which were probably recombinant diploids. Such diploid sectors were individually isolated on MM and then transferred to 4 points of 10 CM plates amended with *p*FA (0.035 mg/mL) to be haploidized. The plates were then incubated for 3-5 days until colored haploid sectors appeared. In order to determine their genotypes, conidia from the edge of these haploid sectors were transferred to 25 defined positions (5 x 5 pattern) of CM plates and incubated for 48 h, and they were then replicated to SM (MM plus 1 µg/mL riboflavin, 0.7 µg/mL *p*-aminobenzoic acid, 0.02 µg/mL biotin, 0.5 µg/mL methionine), SM lacking individually riboflavin, *p*-aminobenzoic acid, biotin and methionine, and SM plus acriflavine (62 µg/mL). After a 48-h incubation at 37°C, the genotypes of the haploid segregants were determined, and the frequency of mitotic crossing-over was then evaluated.

Homozygotization index evaluation

The frequency of mitotic crossing-over was determined by calculating the HI, which was according to Pires and Zucchi (1994) and briefly reviewed by Stoll et al. (2008).

RESULTS

For each herbicide treatment of the original diploid strains (UT448//UT196 and *Dp* II-I//UT196) prior to HI evaluation, these diploids were haploidized. Diuron treatment increased the HI values for both diploids. The most substantial results were found in treatment with a concentration of 2.5 µg/mL. Diploid UT448//UT196 showed an increase in HI values for *ribo*, *paba* and *bi* markers (Table 2A, Figure 1A), while in diploid *Dp* II-I//UT196, the effect of herbicide treatment was noticed scattered all over the markers analyzed (Table 2B, Figure 1B). The markers *Acr*R and *meth*, from the UT448//UT196 diploid, showed similar HI in the control group after treatments with concentrations of 0.25, 2.5 and 25 µg/mL and decreased at higher concentrations (75.0 and 250.0 µg/mL). In fact, a similar pattern was found for *Dp* II-I diploid, where HI for these markers (*Acr*R and *meth*) was smaller at the highest dose (250.0 µg/mL).

Table 2. Homozygotization index (HI) of UT448//UT196 (A) and *Dp* II-I//UT196 (B) diploid strains under diuron treatments.

A.					
Dose (µg/mL)	HI UT448//UT196 (genetic markers)				
	ribo	*paba*	*bi*	*Acr*R	*meth*
Control	1.50	2.33	2.33	9.00	9.00
0.25	7.00	7.00	7.00	8.00	8.00
2.5	10.00	10.00	10.00	11.00	11.00
25.0	2.00	2.00	2.00	12.00	12.00
75.0	3.00	3.00	3.00	5.00	5.00
250.0	3.00	3.00	3.00	2.33	2.33
B.					
Dose (µg/mL)	HI *Dp* II-I//UT196 (genetic markers)				
	ribo	*paba*	*bi*	*Acr*R	*meth*
Control	1.00	1.22	1.22	3.00	5.67
0.25	6.00	9.50	9.50	6.00	6.00
2.5	31.00	31.00	9.67	32.00	32.00
25.0	3.40	3.40	4.50	2.67	3.40
75.0	4.38	4.38	2.91	9.75	5.14
250.0	6.75	5.20	4.17	1.38	2.44

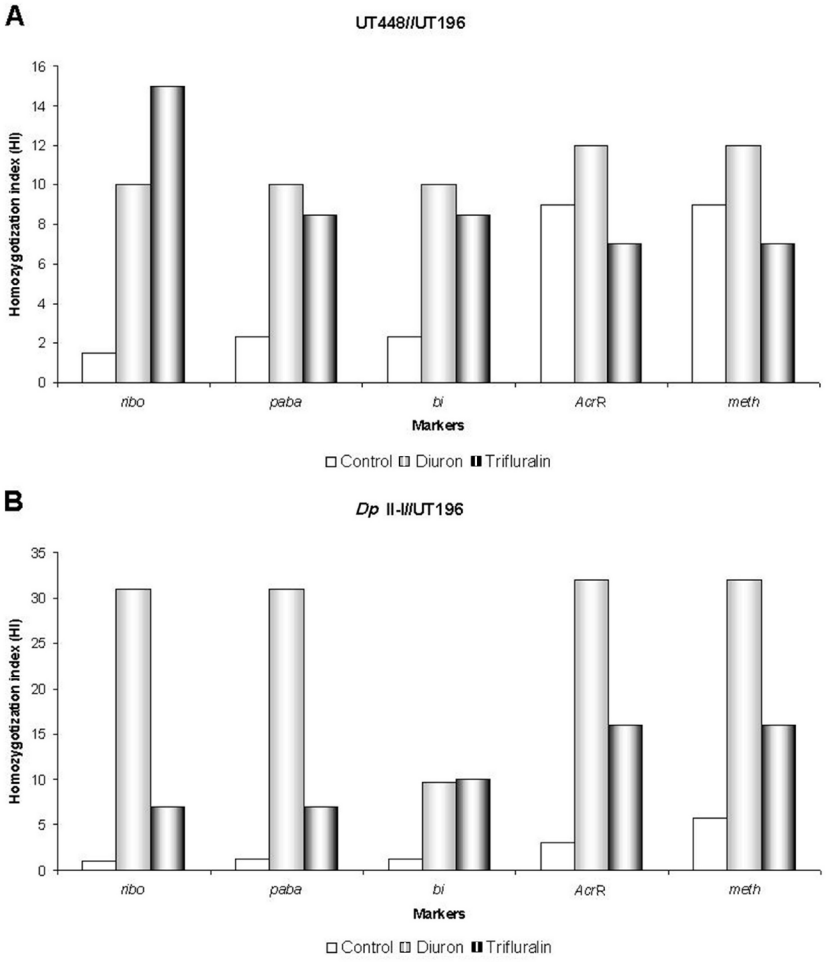

Figure 1. Maximum homozygotization index found after herbicide treatment.

In general, HI for all markers increased after trifluralin treatment. In the diploid UT448//UT196, trifluralin treatment at a concentration of 2.5 µg/mL was also able to induce the highest recombination rates for *paba* and *bi* markers. However, the highest HI for *ribo* marker was found at a concentration of 25.0 µg/mL (Table 3A, Figure 1A). Similar to those results obtained for diuron treatment of UT448//UT196 diploid, *Acr*R and *meth* showed a recombination rate smaller than that observed in control. In fact, *Acr*R and *meth* markers had HI higher than expected for control experiments of this diploid. Unfortunately, a general explanation for this observation is presently not possible on account of the limitations of the present experiments. The segregation rates found for *Dp* II-I//UT196 diploid treated with trifluralin were, in general, smaller than those found for diuron treatment. Also, a higher concentration was necessary to obtain the highest HI for *ribo*, *paba* and *bi* markers (Table 3B, Figure 1B). *Acr*R and *meth* showed the same pattern found for diuron treatment.

Table 3. Homozygotization index (HI) of UT448//UT196 (A) and *Dp* II-I//UT196 (B) diploid strains under trifluralin treatments.

A.					
Dose (μg/mL)	HI UT448//UT196 (genetic markers)				
	ribo	*paba*	*bi*	*Acr*R	*meth*
Control	1.50	2.33	2.33	9.00	9.00
0.25	1.29	1.00	1.29	7.00	7.00
2.5	8.50	8.50	8.50	1.71	1.71
25.0	15.00	3.00	3.00	7.00	7.00
75.0	3.25	3.25	3.25	3.25	3.25
250.0	3.67	3.67	3.67	3.67	3.67
B.					
Dose (μg/mL)	HI *Dp* II-I//UT196 (genetic markers)				
	ribo	*paba*	*bi*	*Acr*R	*meth*
Control	1.00	1.20	1.20	3.00	5.67
0.25	7.00	7.00	4.33	15.00	7.00
2.5	3.25	3.25	3.25	16.00	16.00
25.0	2.50	2.50	3.67	13.00	13.00
75.0	2.67	4.50	10.00	10.00	10.00
250.0	7.50	7.50	4.67	3.25	2.40

Therefore, as homozygotes for conidial color marker are not selected in MM (any color conidia survive on MM, which did not lead to a segregation rate distortion), these markers were not included in this analysis.

Based on the results presented herein, two gene sets can be distinguished according to their patterns. The first one involves the *ribo*, *paba* and *bi* markers, which showed very similar HIs in the two diploid strains. This suggests that they segregate together, without mitotic crossing-over events between them. The other involves the *Acr*R and *meth* markers of chromosome II. Even though the herbicide concentration needed to induce the highest segregation rates for both diploids was usually the same (2.5 μg/mL), the values found for *Dp* II-I//UT196 were higher for all markers analyzed. This finding demonstrates the sensitivity of this diploid for recombinogenic analysis.

DISCUSSION

Indiscriminate use of pesticides has been described as being responsible for causing several hazardous problems with regard to the environment and human health (Pingali and Gerpacio, 1998). Diuron and trifluralin became two of the most used herbicides worldwide, and often have been associated with poisoning and environmental contamination (McMahon et al., 2005; Fernandes et al., 2009). Nevertheless, many studies have demonstrated genotoxic properties for both herbicides (Ebert et al., 1992; Giacomazi and Cochet, 2004), and therefore, some countries banned their use (Konstantinou and Albanis, 2004; Könen and Çavas, 2008). Although both herbicides have shown noxious effects after being evaluated by many biosafety assays, some of these tests did not demonstrate any genotoxic hazards to human beings (Garriott et al., 1991). These singular discrepancies have been explained by non-applicability between the tested-compound and bioassay methods used (Uhl et al., 2003), and therefore, this suggests the importance of analyzing the suspicious substances in several biological systems.

The use of heterozygous diploids of *A. nidulans* has proven to be a feasible alternative to evaluate induced genetic events after treatment with some hazardous agents (Sousa et

al., 2009). By this assay, both herbicides were able to induce mitotic crossing-over in diploid strains of *A. nidulans* and the most substantial results were obtained in *Dp* II-I//UT196 diploid strain after diuron treatment. The HI has already been applied to evaluate the genotoxicity of organic compounds, and the findings presented herein corroborate the previously described sensitivity to detect such events (Salvador et al., 2008).

In general, the maximum HI value found for UT448//UT196 and *Dp* II-I//UT196 diploid strains was obtained at a concentration of 2.5 µg/mL, for both herbicides. Interestingly, this concentration has been commonly reported in soil contaminated with diuron and trifluralin (Moorman and Koskinen, 1990; Garriott et al., 1991). In treatments with higher herbicide concentration there was a decrease in HI values. This pattern is in line with findings by Kunz et al. (1985) who demonstrated that higher doses of genotoxic agents did not correspond to higher recombination rates. Probably, the highest doses of diuron and trifluralin caused more cytotoxic effects (e.g., cell death) than recombinogenic events.

Even though trifluralin treatment was shown to be recombinogenic in *A. nidulans* cells, the HI values found were lower than that of the other herbicide. Hence, this observed pattern could have been influenced by trifluralin's ability to inhibit mitotic cell division (Fernandes et al., 2007, 2009). In fact, compounds known to be anti-carcinogenic, such as isoflavanoids from soybeans, could interfere in the homozygotization process preventing homozygote formation, which leads to elevated HI values (Zucchi, 2006). Accordingly, due to the well-known genotoxic nature of trifluralin and the HI values obtained, the discrepancies between the two herbicides were basically due to their recombinogenic property, and their mitotic inhibition pattern apparently did not interfere with the results.

As demonstrated herein, a bioassay focusing on mitotic recombination of *A. nidulans* was efficient in detecting the genotoxic effects of diuron and trifluralin. As mitotic crossing-over has been related to carcinogenesis in higher eukaryotic cells (Gupta et al., 1997), the HI test proved to be a powerful tool to evaluate suspicious recombinogenic pesticides and should be taken into consideration in further toxicology studies of herbicides.

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