



Anais da Academia Brasileira de Ciências

ISSN: 0001-3765

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Academia Brasileira de Ciências

Brasil

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Anais da Academia Brasileira de Ciências, vol. 82, núm. 4, 2010, pp. 925-932
Academia Brasileira de Ciências
Rio de Janeiro, Brasil

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Anais da Academia Brasileira de Ciências (2010) 82(4): 925-932
(Annals of the Brazilian Academy of Sciences)
ISSN 0001-3765
www.scielo.br/aabc

Ability of *Allium cepa* L. root tips and *Tradescantia pallida* var. *purpurea* in *N*-nitrosodiethylamine genotoxicity and mutagenicity evaluation

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Manuscript received on November 3, 2009; accepted for publication on July 14, 2010

ABSTRACT

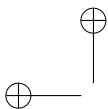
N-nitroso compounds, such as *N*-nitrosodiethylamine (NDEA), can be formed by the reaction of secondary amines with nitrosating agents, and are suspected to be involved in tumors in humans. NDEA has been considered a weak carcinogen in genotoxic assays probably due to the inefficient nitrosamine activation system that is used and/or the efficient repair system. In this work, we evaluated the sensibility of *Allium cepa* L. root tips and *Tradescantia stamen* hair mutation assay (Trad-SH) using *Tradescantia pallida* var. *purpurea* for NDEA (0.1; 0.5; 5 and 25mM) genotoxicity and mutagenicity induction. *Allium cepa* L. was treated with different NDEA concentrations for 3h, 4 and 3 consecutive days, including negative control (distilled water) and positive control maleic hydrazide (MH 30µg/mL). After treatment, the roots were hydrolyzed, squashed, and the mitotic index (MI) and cytological abnormalities were scored. The results revealed a cytostatic effect of NDEA (0.5 and 5mM), showing a significant reduction in the MI. Chromosome stickiness suggests a NDEA toxic effect. *T. pallida* purpurea did not respond to mutagens with a dose-dependent pattern. In conclusion, our study indicates that the root tips of *Allium cepa* L. have sensibility to detect NDEA genotoxicity, but not for Trad-SH test.

Key words: *Allium cepa*, genotoxicity, mutagenicity, *N*-nitrosodiethylamine, *Tradescantia pallida* var. *purpurea*.

INTRODUCTION

Studies on the mechanisms of the cancer induction process have revealed that specific chemical compounds are able to produce diseases by themselves (Miller 1970, Aiub et al. 2006). *N*-nitroso compounds, such as *N*-nitrosodiethylamine (NDEA), can be formed by the reaction of secondary amines with nitrosating agents, and are suspected to be involved in tumors in humans (Lijinsky 1992, Aiub et al. 2006). The importance of the analytical determination of the compounds is owing to the carcinogenicity of nitrosamines and their formation. *N*-nitrosamines can be found in treated wastewater effluents, as well as in ground and drinking water (Char-

nant formation pathways involved a nitrosation reaction between monochloroamine and dimethylamine derivatives, both mutagenics (Mitch and Sedlak 2000, and Valentine 2003, Jurado-Sánchez et al. 2009). NDEA is activated by cytochrome P450 enzymes and produces detectable results in the ethylation of *N*⁷ and in the oxidation of guanine (Pegg 1983, Aiub et al. 2004). A lower level of ethylation is also observed at the oxidation of thymine (Saffhill et al. 1985, Aiub et al. 1999). (Wild and Montesano 1991, Nakatsuru et al. 1999). Precarcinogenic lesions *O*⁶-ethylguanine and *O*⁶-methylthymine, if not repaired, will lead to mutation and tumor formation in superior animals (Pegg 1983, and Montesano 1991, Aiub et al. 2004). Nitrosamines



Zeiger et al. 1987, Aiub et al. 2004). The reason for these can be due to the authors usually testing NDEA at much higher concentrations than those expected *in vivo* (Simmon 1979, Aiub et al. 2003). The apparent inconsistency in NDEA results presented in many papers could be attributed mainly to the inefficient nitrosamine activation system used and/or to the efficient repair system (Yahagi et al. 1977, Mohn 1981, Prival and Mitchell 1981, Aiub et al. 2003).

The mutagenicity and genotoxicity evaluation, using more than one test system, is often pointed out. One of the main reasons for using several tests is either to investigate whether different biological models and cell types differ in their sensitivity to environmental chemicals, and to obtain information concerning the predictive value of short-term tests (Fiskesjö 1981). Plant test systems are important to detect mutagens in different environments, since they combine a high sensibility and a great capacity to evaluate distinct genetic endpoints, from point mutations to chromosomal aberrations, either in individual cells or in organs such as leaves, pollens and endosperm (Grant 1994).

The species *Allium cepa* presents advantages, including low raising costs, easy handling, and suitable chromosomal features. This plant bears large and few chromosomes ($2n = 16$), which facilitates the evaluation of chromosome damages and/or disturbances in cell division cycle, including eventual aneuploidy risks (Grant 1982, Fiskesjö 1985, Morais Leme and Marin-Morales 2008). Root tips of *Allium cepa* L. have been recommended as a standard for cytogenetic assay in environmental monitoring due to the correlation of these plants with mammalian and non-mammalian test systems (Constantin and Owen 1982, Cauhan et al. 1999, Nilüfer et al. 2008).

The *Tradescantia* stamen hair mutation assay (Trad-SH) was developed by Dr. Arnold H. Sparrow and colleagues during the late 1950s and throughout the 1960s at the Brookhaven National Laboratory, Long Island, NY. The Trad-SH is a simple, cost effective short-term bioassay that yields data within 11-14 days, and is easily adapted for either in house or *in situ* moni-

in this assay are the mitotic stamen hair cells developing in the young flower buds. The traditional assay is based on the fact that these stamen hair cells in *Tradescantia* clone 4430 are heterozygous, being the phenotypic change in pigmentation ranging from blue (dominant) to pink (recessive) among blue cells, which is a single mutant event (Ma et al. 1994). The genotype of *Tradescantia pallida* var. *purpurea* concerning the cells that are present in stamen hair is different from the one of *Tradescantia* clone 4430, although presenting equal clastogenic sensibility for micronuclei induction (Suyama et al. 2002).

Tradescantia pallida var. *purpurea* is tetraploid, exhibits a notable resistance to parasites and insects, is spread quite easily, and is widely distributed in the streets and gardens of the Brazilian cities (Suyama et al. 2002).

The aim of the present study was to evaluate the sensibility of root tips of *Allium cepa* L. and Trad-SH assay test using *Tradescantia pallida* var. *purpurea*, for NDEA genotoxicity and mutagenicity.

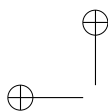
MATERIALS AND METHODS

CHEMICAL PRODUCTS

In this study, it was used NDEA (CAS no.: 55-18-5), chemical formula $C_4H_{10}N_2O$, molecular weight 102.1, maleic hydrazide (MH) (CAS no.: 123-33-1), chemical formula $C_4H_4N_2O_2$, molecular weight 112.09; all of them obtained from Sigma (St. Louis, Mo, USA).

ROOT TIPS OF *Allium cepa* L.

Onion bulbus (*Allium cepa* L., $2n = 16$) were obtained commercially and were placed in small jars with the basal ends dipping in distilled water. The new emerged roots (1-2 cm in length) were treated with a series of NDEA concentrations (0.1; 0.5; 5 and 25Mm) for 3h, for 3 consecutive days, and diluted with distilled water. For the negative control it was used distilled water and, for the positive control, MH 30 μ g/mL. The NDEA treatment and control roots that were exposed were removed from the bulbs and hydrolyzed with a solution composed of 9 parts of 45% acetic acid and 1 part



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1999). Three independent assays were performed for each treatment, and scoring was done from the three roots in triplicates. The MI (Mitotic Index) was calculated for each treatment as a number of dividing cells/100 cells. The cytological abnormalities (anaphase bridges, laggards, micronuclei and stickiness) were scored in the mitotic cells. Statistical analysis – one-way analysis of variance (ANOVA) – was used as described elsewhere (Aiub et al. 2004).

Tradescantia STAMEN HAIR MUTATION ASSAY (TRAD-SH)

The assessment of *in situ* mutagenesis involved exposure of *Tradescantia pallida* var. *purpurea* in pots containing a 3:1 mixture of sieved soil and vermiculite. The plants were watered three times a week and received 100mL of Hoagland nutrient solution, twice per month. The treatment was done with NDEA at concentrations: 0.1; 0.5; 5 and 25mM for 3 consecutive days. As negative control it was used distilled water, and as positive controls MH (30 μ g/mL) and X-ray (100 cGy) by means of a therapeutic X-ray device (Pantak Seifert), operated at 50kV, 30mA. After 7-14 days of treatment, 10 appropriate young inflorescences from each sample were used, and the stamens of the recently opened flowers were cut and placed on a slide. Hairs and mutation events were counted under 40X magnification. A mutation event consists of one or a sequence of adjacent white cells among the dominant pink cells, in any position of the stamen hair. Statistical analysis – one-way analysis of variance (ANOVA) – was used as described elsewhere (Aiub et al. 2004).

RESULTS

ROOT TIPS OF *Allium cepa* L.

The MI and, consequently, the frequency of mitotic phases are given in Table I for cells of *Allium cepa* treatments with NDEA at concentrations 0.1mM; 0.5mM; 5mM and 25mM.

The *Allium cepa* cell roots treated with NDEA (0.5mM and 5mM) showed a decrease in the MI ($p <$

number of cells in prophase and metaphase, especially for NDEA at 0.5mM, which indicates later in the cell cycle. NDEA treatments induced a wide range of mitotic abnormalities in the root tips of *A. cepa*. The types and percentage of these abnormalities are given in Table II and represented in Figure 1 as anaphase bridges, laggards, micronuclei and stickiness.

We observed a significant increase in the frequency of abnormalities for all NDEA concentrations compared with the negative control ($p < 0.05$). Anaphase bridges (8%) were verified only at NDEA 0.5mM, although at all concentrations, lagging chromosome was observed. We detected the occurrence of micronuclei for all NDEA concentrations, especially at 0.5 and 5mM, increasing 25% and 30%, respectively ($p < 0.05$). Chromosome stickiness (37%) increases for all NDEA concentrations ($p < 0.05$ at 25 mM).

Tradescantia STAMEN HAIR MUTATION ASSAY (TRAD-SH)

Mutation frequencies were obtained in *Tradescantia pallida* var. *purpurea* exposed to NDEA at different concentrations (Table III).

We do not observe a significant result even when compared with negative and positive controls.

DISCUSSION

The root tips of *Allium cepa* L. have often been used for the determination of cytotoxic (Vicentin et al. 1999), cytostatic and/or genotoxic effects of some substances (Nilüfer et al. 2008). Moreover, for *in situ* mutagenesis, meristematic cells of *Allium cepa* L. were considered efficient cytogenetic material for detection of mutagenic activity of the environmental chemicals helping cancer research (Levan 1951, Ma et al. 1995, Ateeq et al. 2002, Nilüfer et al. 2008). NDEA is activated by cytochrome P450 enzymes (Pegg 1983, Aiub et al. 2004). These enzymes play an important role in the detoxification process in higher plants as well (Werck et al. 1999, Sandermann 1992, Riffat and Masood 2006). Previous studies have shown an interest in the mechanism of cytochrome P450 dependent mixed function



TABLE I
Mitotic index and the percentage of mitosis stages in the root tip cells of *A. cepa* treated with different NDEA concentrations.

Concentration (mM)	Mitotic index	% Prophase	% Metaphase	% Anaphase	% Telophase
Negative Control	9.88 ± 0.25	50	30	10	10
0.1 mM	8.04 ± 0.01	30	30	20	20
0.5 mM	1.13 ± 0.98*	18*	10*	36	36
5 mM	1.70 ± 0.26*	15*	55	15	15
25 mM	5.23 ± 2*	18*	36	5	41
Positive Control	0.34 ± 0.76	100	0	0	0

Negative Control: distilled water. Positive Control: MH 30µg/mL. Statistical analysis related to negative control * $p < 0.05$.

TABLE II
Mitotic aberrations in the root tip cells of *A. cepa* treated different NDEA concentrations.

Concentration (mM)	% Total abnormalities	% A.B.	% L.	% MN.	% S.
Negative Control	0	0	0	0	0
0.1 mM	59*	0	23	6	30*
0.5 mM	74*	8	16	25*	25*
5 mM	75*	0	15	30*	30*
25 mM	79*	0	23	19	37*
Positive Control	100	0	0	100	0

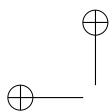
A.B., anaphase bridges; L., laggards; MN., micronucleus; S., stickiness. Negative Control: distilled water. Positive Control: MH 30µg/mL. Statistical analysis related to negative control * $p < 0.05$.

et al. 1987, Aas et al. 2001, Bard et al. 2002, Moore et al. 2003, Riffat and Masood 2006).

Our findings reflect the utility of root meristem cells of *Allium cepa* L. for monitoring the genotoxic effects of NDEA as reported to other nitroso compound (Fiskesjö 1981). We demonstrated NDEA cytostatic effect (0.5 and 5mM) through a significant reduction in the mitotic index (MI) in a dose dependent manner (Table I). The delay observed in mitosis (Table I) could be due to the inhibition of DNA synthesis (Schneiderman et al. 1971, Sudhakar et al. 2001, Türköglu 2007) or to a blocking in the G2-phase of the cell cycle, preventing mitosis (Van't Hof 1968, Türköglu 2007).

eral spindle-related abnormalities like laggarding chromosomes, stickiness and micronuclei. Our results show that NDEA treatment for all concentrations presents a significant higher mitotic aberrations compared with the control group ($p < 0.05$), and chromosome stickiness was the main alteration (Table II). NDEA induced a significant increase of chromosome stickiness in all concentrations, which indicates high toxic effect. Other nitroso compounds like N-Methyl-*N'*-Nitro-N-Nitrosoguanidine (MNNG) and 4,6-Dinitro-O-Cresol also showed stickiness effect using the same method (Fiskesjö 1981, Nölüfer et al. 2008).

NDEA at 0.5 and 5mM showed a significant in-



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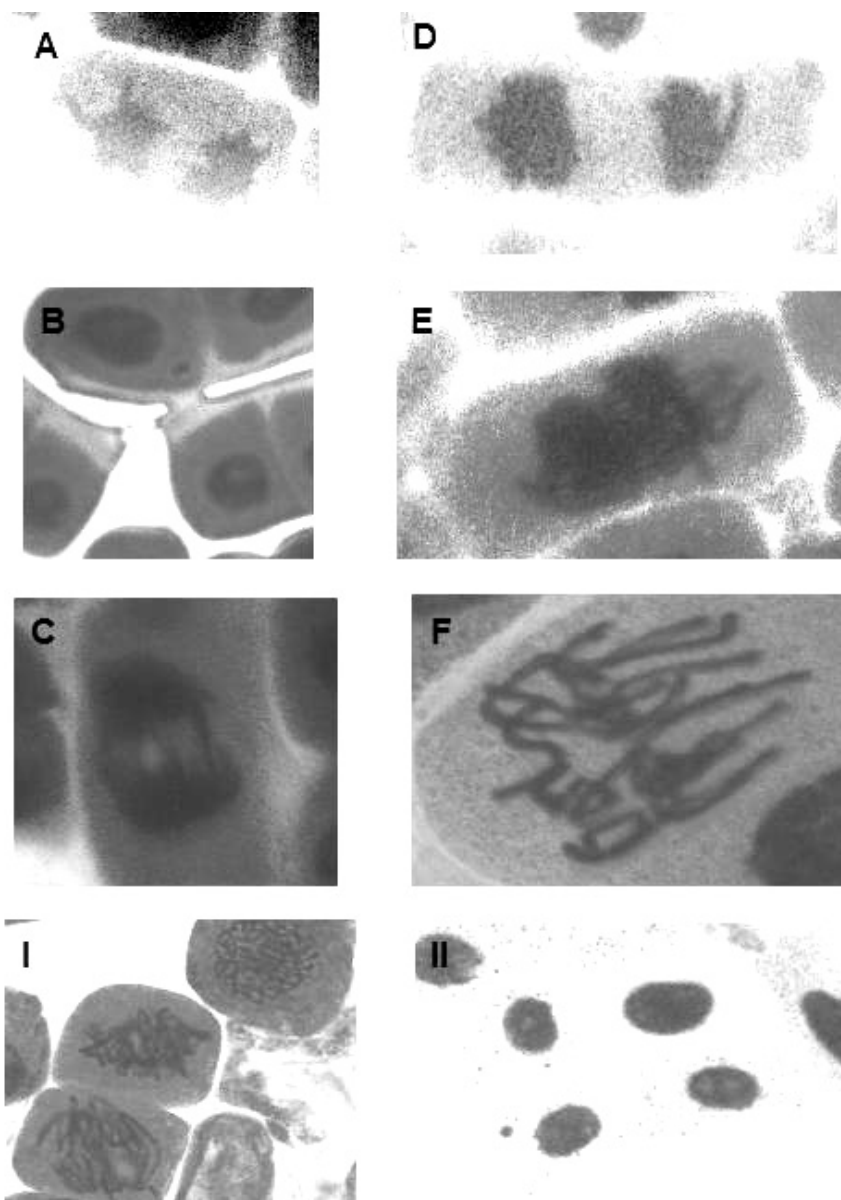


Fig. 1 – Different types of aberration induced by NDEA in *Allium cepa* root tips: (A) lagging chromosome at anaphase (25mM); (B) micronucleus at interphase (5 mM); (C) bridge formation (0.5 mM); (D) lagging chromosome at anaphase (0.5 mM); (E) stickiness formation (0.1 mM); (F) chromosome break at metaphase (0.1 mM). (I) normal prophase, metaphase and anaphase induced by negative control (water); (II) micronucleus and high synthesis of protein induced by positive control (MH 30µg/mL).

mitotic cells. Besides, micronucleus formation implies in loss of genetic material and DNA breakage, which

and chromatids (Türköglu 2007). Besides, lagging chromosome was due to the failure of the chromoso



TABLE III
Frequency of mutation (mutations/100) in stamen hairs of *Tradescantia pallida* var. *purpurea* treated with different NDEA concentrations.

Concentration (mM)	SH frequency (Mean \pm S.D.)
Negative Control	1.4 \pm 0.005
0.1	0.84 \pm 0.19
0.5	1 \pm 0.01
5	0.79 \pm 0.37
25	1.04 \pm 0.01
MH _a	1.03 \pm 0.6
100 cGY _{aa}	1.01 \pm 0.01

Negative Control: distilled water. _aPositive Control: MH 30 μ g/mL; _{aa}Positive Control: X-ray 100 cGY.

* $p < 0.05$.

and unequal chromosome distribution were observed for all NDEA concentrations, but no significant increase was observed compared with the negative control.

Our results in root tips of *Allium cepa* L. test are in agreement with NDEA genotoxicity and mutagenicity using SOS chromotest and Ames test, respectively. We verified, using SOS chromotest, an induction of *sfIA* expression in the *uvrA* *Escherichia coli* tester strains (PQ35, PQ37 PQ65 and PQ66) that occurred at doses between it 0.75 and 36.46 μ g/ml, with S9 mix and in PQ66, without the S9 mix, for 36.46 μ g/ml of NDEA concentration. In Ames test, was detected a mutagenic response in *Salmonella typhimurium* strains TA98 (NDEA 25.47 μ g per plate) and TA100 (NDEA 5.09 μ g per plate) in the presence of the S9 mix with pre-incubation of 90 min (Aiub et al. 2003). As the NDEA concentrations used in *S. typhimurium* Ames test (μ g/mL) are much lower than others used for *Allium cepa* assay (mM), we suggest that high concentrations can induce chromosomal aberrations including DNA breaks, as demonstrated for MN. Other possibility is the ability of superior plants to possess the integrity of cytochrome P450, which increases the generated metabolites.

One of the methods most widely used to detect genotoxic activity of chemical or physical agents is the

(Aa). The dominant allele (A) accounts for the blue color, and the recessive allele (a) is responsible for the pink color. Mutation in the dominant allele will result in the expression of the recessive allele (pink cells) (Ma et al. 1994), (Moya-Alvarez et al. 2001). We supposed that *Tradescantia pallida* var. *purpurea* has recessive white cells and domination pink cells. This characteristic is different in the clone 4430, which has pink cells recessive and domination blue cells (Ma et al. 1994). The choice of *Tradescantia pallida* var. *purpurea* test was based on two criteria generally taken into consideration in biomonitoring: its wide distribution in Rio de Janeiro and its easy propagation. As the first step in our study, we decided to test the sensitivity of *T. pallida purpurea* to known mutagens, like NDEA, and to test its capacity to respond in terms of mutagenic events (characterized by color change) formed in stamen hair. The results depicted in Table III clearly indicate that *T. pallida purpurea* did not respond to NDEA induced DNA damage with a dosedependent pattern. These results are similar to the ones of other works that have been reported with *Tradescantia* staminal hairs (clone 4430) using 1, 5 and 10 mM (Moya-Alvarez et al. 2001).

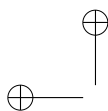
In conclusion, our study indicated that the root tips of *Allium cepa* L. have sensibility to detect NDEA genotoxicity. However, Trad-SH test using *T. pallida purpurea* is not sensitive to detect NDEA mutagenicity.

ACKNOWLEDGMENTS

This work was supported by Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Sub-Reitoria de Pesquisa da Universidade do Estado do Rio de Janeiro (SR2-UERJ).

RESUMO

Nitrocompostos, como *N*-nitrosodietilamina (NDEA), podem ser formados pela reação entre uma amina secundária e agentes nitrosantes e são suspeitos de estarem envolvidos na formação de tumores em humanos. NDEA é considerada um



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avaliamos a sensibilidade de ensaios com *Allium cepa* L. e *Tradescantia pallida* var. *purpurea* (Trad-SH) à genotoxicidade e mutagenicidade induzidas por diferentes concentrações de NDEA (0,1; 0,5; 5 e 25mM) por 3h, por 3 dias consecutivos, incluindo controle negativo (água destilada) e controle positivo, hidrazida maleica (MH 30µg/mL). Depois do tratamento, as raízes foram hidrolizadas, esmagadas e o índice mitótico (IM) e anormalidades citológicas foram contadas. Os resultados revelaram um efeito citostático de NDEA (0,5 e 5mM), pela significativa redução do IM. *Chromosome stickiness* sugere um efeito citotóxico de NDEA. *T. pallida purpurea* não respondeu ao mutágeno com um padrão dose dependente. Em conclusão, nossos estudos indicaram que raízes de *Allium cepa* L. possuem sensibilidade na detecção genotóxica de NDEA, mas não para o ensaio Trad-SH.

Palavras-chave: *Allium cepa*, genotoxicidade, mutagenicidade, *N*-nitrosodietilamina, *Tradescantia pallida* var. *purpurea*.

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