UNIVERSIDADE ESTADUAL DE SANTA CRUZ

PROGRAMA DE PÓS-GRADUAÇÃO EM GENÉTICA E BIOLOGIA MOLECULAR

ESTUDOS COMPARATIVOS DE SENSIBILIDADE E MUTABILIDADE NOS FUNGOS *Saccharomyces cerevisiae* E *Moniliophthora perniciosa*

TATIANA SETENTA BASSO

ILHÉUS – BAHIA – BRASIL

Março de 2008

TATIANA SETENTA BASSO

ESTUDOS COMPARATIVOS DE SENSIBILIDADE E MUTABILIDADE NOS FUNGOS *Saccharomyces cerevisiae* E *Moniliophthora perniciosa*

Dissertação apresentada à Universidade Estadual de Santa Cruz, como parte das exigências para obtenção do título de Mestre em Genética e Biologia Molecular.

Área de concentração: Genética e Biologia Molecular

ILHÉUS – BAHIA – BRASIL

Março de 2008

TATIANA SETENTA BASSO

ESTUDOS COMPARATIVOS DE SENSIBILIDADE E MUTABILIDADE NOS FUNGOS *Saccharomyces cerevisiae* E *Monilophthora perniciosa*

Dissertação apresentada à Universidade Estadual de Santa Cruz, como parte das exigências para obtenção do título de Mestre em Genética e Biologia Molecular.

Área de concentração: Genética e Biologia Molecular

APROVADA:

Prof. Dra. Jane Marlei Boeira
(UERGS / RS)

Prof. Dr. Diego Bonatto
(UCS / RS)

Prof. Dra. Aline Silva
(UESC / BA)

Prof. Dr. Martin Brendel
(UESC / BA – Orientador)

A minha família em especial a minha mãe, pelo carinho e amor incondicional dedicados em todos os momentos de minha vida,

Dedico

AGRADECIMENTOS

À Deus acima de tudo, por tudo que sou, por tudo que tenho e por toda luz e força concedidas durante essa minha jornada.

Ao professor Martin Brendel, meu orientador e a professora Cristina Pungartnik, por toda atenção e pela grande oportunidade de aprendizado, que se iniciou mesmo antes deste mestrado, pela confiança e apoio dados ao meu trabalho e a minha pessoa, o meu muito obrigado!

Ao professor Júlio Cascardo pelo apoio dado durante a execução deste projeto.

À Universidade Estadual de Santa Cruz e a todos os colegas e professores do Programa de Pós-Graduação em Genética e Biologia Molecular da UESC, obrigada pela amizade, companheirismo e aprendizagem.

À FAPESB, a CAPES e a MARS que possibilitaram a realização deste trabalho.

À Luciana, secretária do programa, por toda a ajuda, gentileza e amizade.

Ao meu amigo e colega de graduação e mestrado Edílson, por todo esse tempo de convivência, de alegrias e tristezas, de risos e de desabafos.

Aos colegas e amigos do Laboratório de Monitoramento Ambiental, em especial à equipe Pungartnik: Sônia, Neide, Gabriele e Wagner, pelo grande convívio que tivemos, pelos momentos alegres e de descontração e por aqueles de aperto e desespero também! Vocês estão em meu coração!

Ao meu namorado Alex, pela paciência, carinho, companheirismo e ombro “amigo”, principalmente na fase terminal de minha dissertação.

À minha família, a minha irmã Julianna, à minha avó Nilza, ao meu pai Vilson, que mesmo de longe torce pelo meu sucesso e principalmente à minha mãe Wilse, sem o seu carinho e apoio eu não estaria aqui!

À todos os meus amigos que direta ou indiretamente contribuíram para a realização deste trabalho.

ÍNDICE

Basso, Tatiana Setenta, M.Sc., Universidade Estadual de Santa Cruz, Março de 2008. **Estudos comparativos de sensibilidade e mutabilidade nos fungos *Saccharomyces cerevisiae* e *Moniliophthora perniciosa*.** Orientador: Dr. Martin Brendel. Co-orientadora: Dra. Cristina Pungartnik. Colaborador: Dr. Júlio C. M. Cascardo.

Os organismos do reino dos Fungos são seres eucariotos que possuem larga utilidade industrial e biotecnológica. Um modelo deste reino extremamente bem caracterizado e usado em aplicações industriais é a levedura *Saccharomyces cerevisiae*. A partir do acúmulo de conhecimento sobre este modelo, é possível realizar estudos de expressão heteróloga de genes do fungo causador da doença vassoura-de-bruxa, *Moniliophthora perniciosa*, que afeta o cacaueiro (*Theobroma cacao*). Através dos estudos de identificação do mecanismo de ação do $SnCl_2$ em *S. cerevisiae,* da análise da influência da ploidia e da autofagia na sensibilidade à mutagênicos em *M. perniciosa* e da obtenção de mutantes deletados em genes definidos de *M. perniciosa*, este trabalho visa contribuir para a elucidação dos possíveis mecanismos de metabolização e consequentes modos de proteção e reparo induzido por agentes mutagênicos nos fungos *M. perniciosa* e *S. cerevisiae*. Uma mutação em um gene que codifica para a proteína Rnr4 em *S. cerevisiae* sensibiliza a levedura ao $SnCl_2$. A sensibilidade ao $SnCl_2$ do mutante *rnr4*Δ é de três a quatro vezes maior quando comparado com a da cepa selvagem, independente da fase de crescimento (LOG ou STAT). Isto significa que outros mecanismos estejam envolvidos, mais provavelmente o não-funcionamento do reparo de DNA causado pelo baixo fornecimento de dNTPs. A ploidia (fase monocariótica/ fase dicariótica) do fungo *M. perniciosa* influencia de maneira diferente quando tratado com os mesmos agentes mutagênicos, nas mesmas doses e também na indução do processo autofágico. Existe um grau de resistência crescente para os agentes que causam estresse oxidativo (H_2O_2, PAQ): basidiósporos > hifas monocarióticas (crescidas em glicerol) > hifas dicarióticas (glicerol) > hifas dicarióticas (crescidas em glicose). Para os agentes que causam danos ao DNA (UVC, 4NQO) observou-se a mesma resistência para os basidiósporos e hifas mono/dicarióticas e somente as hifas

dicarióticas (glicose) apresentaram maior sensibilidade. Testes para a avaliação da capacidade de inibição do tubo de germinação dos basidiósporos frente aos mesmos agentes mutagênicos mostraram que não existe correlação entre formação do tubo germinativo e viabilidade do fungo. Mutações em *M. perniciosa* podem ser induzidas por agentes químicos ou físicos e selecionadas através da resistência de mutantes ao agente tóxico canavanina (análogo da arginina). Mutantes resistentes à canavanina (can^R) foram isolados a partir da mutagenização de basidiósporos de *M. perniciosa*, demonstrando que a utilização dos mesmos conceitos genéticos do fungo modelo *S. cerevisiae* é factível. Um dos mutantes de *M. perniciosa* can^R apresentou a mesma resistência ao UVC que a linhagem selvagen*,* mas foi muito mais resistente ao H_2O_2. Os mecanismos que envolvem esta resposta ainda não foram elucidados.

Palavras-chave: *Moniliophthora perniciosa*, *Saccharomyces cerevisiae*, sensibilidade, mutagênese

Basso, Tatiana Setenta, M.Sc., Universidade Estadual de Santa Cruz, March of 2008. **Comparative studies of sensitivity and mutagenesis in the fungi *Saccharomyces cerevisiae* and *Moniliophthora perniciosa*.** Advisor: Dr. Martin Brendel. Adivisor Committee Members: Dra. Cristina Pungartnik and Dr. Júlio C. M. Cascardo.

Organisms of the kingdom of the Fungi are eukaryotes that have vast industrial and biotechnology applications. The yeast *Saccharomyces cerevisiae* is an extremely well-characterized fungus. Based on the accumulation of knowledge on this model, it is possible to project and carry out studies of gene expression in a more complex fungal system, the phytopathogenic basidiomycete *Moniliophthora perniciosa* that causes witches' broom disease, severely affecting the cacaueiro (*Theobroma cacao*). Combining studies for the identification of the mechanism of action of $SnCl_2$ in *S. cerevisiae,* with the analysis of the influence of ploidy and autofagia on sensitivity to mutagens in *M. perniciosa* and obtaining mutagen-induced mutants in *M. perniciosa,* this work aims to contribute to the elucidation of the possible contributions of general metabolism and specific modes of protection and repair on mutagen-induced damage in both fungi. A mutation in a gene that encodes protein Rnr4p in *S. cerevisiae* leads to sensitivity to $SnCl_2$. The *rnr4Δ* mutant strain is 3-4x more sensitive to $SnCl_2$ as compared with the wild type, regardless of the culture´s growth phase (LOG or STAT), indicating that growth phase is not decisive in enhancing sensitivity. Thus, other mechanisms are involved, most probably the non-fully functional repair of $SnCl_2$-induced DNA damage, caused by the low supply of dNTPs. Ploidy phase (monokaryotic/ dikaryotic) of the fungus *M. perniciosa* has pronounced influence on cell sensitivity after equal treatment with the same mutagenic agents. It also influences the process of induction of autophagy. There is a growing degree of resistance to the agents that cause oxidative stress (H_2O_2, PAQ) with the following ranking: basidiospores > hyphae monokaryotic (glycerol- grown) > hyphae dicakaryotic (glycerol-grown) > hyphae dicakaryotic (glucose- grown). Basidiospores and monokaryotic/dikaryotic (glycerol-grown) hyphae had the same resistance DNA damaging agents UVC and 4NQO, while dikaryotic glucose-grown hyphae were

more sensitive. Tests assessing the ability to inhibit formation of the germination tube of the basidiospores showed that there is no correlation between inhibition of germination tube formation and the viability of the fungus at chronic mutagen exposure. Mutations in *M. perniciosa* can be induced by physical or chemical agents and selected via resistance of the mutants to the toxic agent L-canavanine (analogue of arginine). Canavanine resistant mutants (can^R) could be isolated from mutagenized *M. perniciosa* basidiospores, applying methods already described for *S. cerevisiae.* One of the can^Rmutants of *M. perniciosa* had the same resistance to UVC as the wild type, but was much more resistant to H_2O_2. The mechanisms involving this differentiated sensitivity response have not yet been elucidated.

Keywords: *Moniliophthora perniciosa*, *Saccharomyces cerevisiae*, mutagen sensitivity, mutagenesis.

4NQO	Óxido de 4-nitroquinoleína
4HAQO	Óxido de 4-hidroxiaminoquinolina
4HQO	Óxido de 4-aminoquinolina
CAN	Canavanina
CO_2	Dióxido de carbono
DNA	Ácido desoxirribonucléico
dNDP	Desoxinucleotídeo difosfato
dNTP	Desoxinucleotídeo trifosfato
ERO	Espécie reativa de oxigênio
H_2O_2	Peróxido de hidrogênio
LOG	Fase logarítmica de crescimento
NDP	Nucleotídeo difosfato
O_2	Oxigênio
$O_2^{\bullet -}$	Ânion superóxido
$OH^{\bullet}$	Radical hidroxil
$^1O_2$	Oxigênio *singlet*
PAQ	Paraquat
RNA	Ácido ribonucléico
$SnCl_2$	Cloreto de estanho
STAT	Fase estacionária de crescimento
UVC	Radiação ultravioleta (254 nm)

1. Introdução e Revisão Bibliográfica

1.1. Os fungos

Os fungos são seres eucariotos podendo ser unicelulares, como as leveduras, ou multicelulares, como os filamentosos ou bolores. A filogenia apresentada para os Eumycota (fungos verdadeiros) reconhece quatro filos: Chytridiomycota, Zygomycota, Ascomycota e Basidiomycota (Bruns *et al*.1991; 1993).

Os fungos são encontrados no solo, na água, nos vegetais e animais, sendo importantes decompositores, como o *Trichosporon* sp (Kaszycki *et al*., 2006) ou parasitas, como algumas espécies de Saprolegnia (Fernández-Benéitez *et al*., 2008). São largamente utilizados na indústria alimentícia, como na produção de pães, vinhos, cervejas (*Saccharomyces cerevisiae*), e biotecnológica, como na produção de antibióticos (penicilina pelo *Penicillium crysogenum*) (Mucheroni e Matias, 1996). Além disso, alguns fungos podem ser patogênicos para ao homem, como *Candida albicans* (Vultaggio *et al*., 2007), para os animais, *Malassezia pachydermatis* (Dizotti e Coutinho, 2007) ou para as plantas, como *Moniliophthora perniciosa* (Griffith, 2004).

1.1.1. A levedura *Saccharomyces cerevisiae*

A levedura *S. cerevisiae* é um fungo ascomiceto bastante estudado e notavelmente semelhante às células de mamíferos no que se refere à presença de macromoléculas, organelas e proteínas com homologia às proteínas humanas, tornando-a um organismo importante nas pesquisas sobre mutagênese, reparo de DNA e mecanismos que respondem ao estresse oxidativo (Costa e Ferreira, 2001).

Foi o primeiro organismo eucarioto a ter seu genoma totalmente sequenciado com um tamanho de 12.068 kb, organizado em 16 cromossomos e média de um gene a cada 2.000 pb (Goffeau *et al.* 1996), mostrando-se extremamente importante para o desenvolvimento de novas técnicas e servindo como referência para análise evolutiva e comparativa de outros organismos eucariotos superiores (Goffeau, 2000; Liti e Louis, 2005; Garfinkel, 2005).

1.1.1.1. O ciclo de vida da levedura *Saccharomyces cerevisiae*

A levedura *S. cerevisiae* possui um ciclo eucariótico bem definido, podendo ser induzida tanto à um ciclo meiótico quanto ao mitótico de crescimento (Figura 1). Suas células se dividem por brotamento logo após a duplicação do seu DNA, com tempo de geração de aproximadamente 120 minutos, para as células haplóides, e 90 minutos, para as diplóides, quando incubadas à temperaturas entre 28-30ºC (Zimmermann *et al.,* 1984).

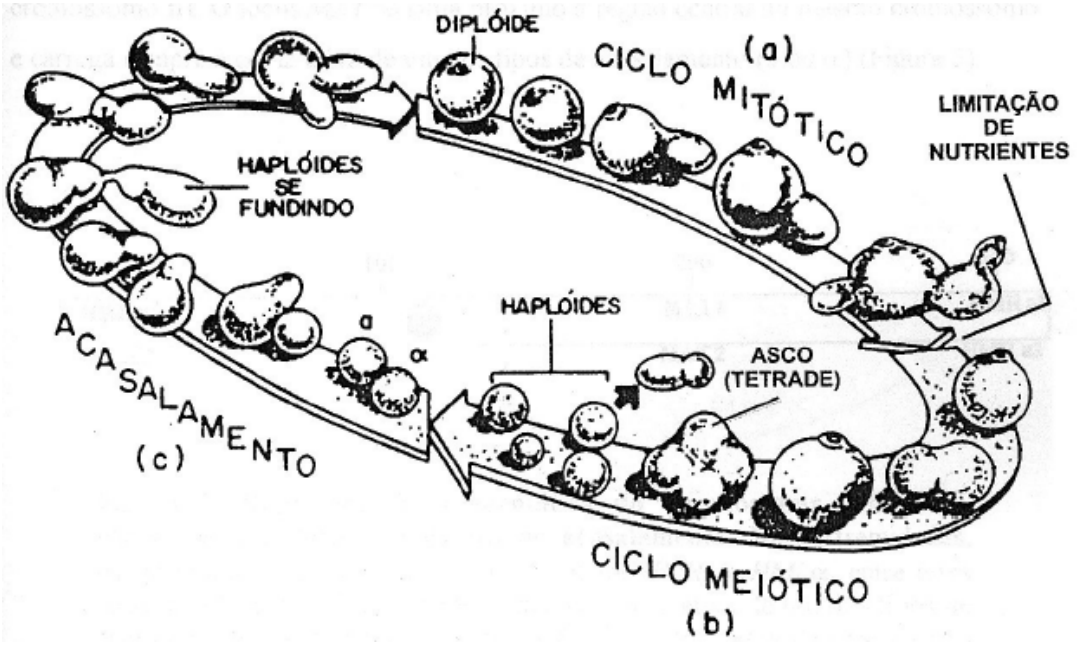


Figura 1. Ciclo de vida da levedura *Saccharomyces cerevisiae* (Adaptado de Zimmermann *et al.,* 1984).

Durante o seu ciclo mitótico (Figura 1a), as células diplóides formam brotos que crescem e se separam de sua célula mãe. As células diplóides podem ser induzidas à meiose e conseqüente esporulação, quando ocorre limitação de nutrientes no meio (Figura 1b). A esporulação origina quatro esporos (haplóides) que se desenvolvem dentro de um ascósporo, que por sua vez se multiplicam por divisão mitótica e brotamento, o que é muito útil, por exemplo, na construção de linhagens geneticamente definidas (Zimmermann *et al.,* 1984). Os esporos podem crescer mitoticamente originando células haplóides ou, então, os esporos haplóides com sinais de acasalamento opostos fundem-se formando uma nova

célula diplóide (Figura 3c) (Kurjan, 1992; 1993). As células diplóides são maiores e mais alongadas e não apresentam a capacidade de acasalamento; as células haplóides são mais arredondadas e liberam fator de acasalamento (Fuge e Werner-Washburne, 1997).

1.1.1.2. O metabolismo celular

O termo metabolismo celular é utilizado para designar o conjunto de todas as reações químicas que ocorrem nas células, sendo estas responsáveis pelos processos de síntese (anabolismo) e degradação (catabolismo) dos nutrientes no interior celular (Nelson e Cox, 2002).

Todos os fungos, a partir de uma fonte de carbono, de nitrogênio, de íons minerais e da água são capazes de sintetizar todos os compostos orgânicos necessários para seu crescimento (Carlile e Watkinson, 1996).

1.1.1.2.1. O metabolismo de carboidratos

A levedura *S. cerevisiae* é capaz de se desenvolver em condições anaeróbicas e aeróbicas de crescimento, sendo assim um organismo anaeróbico facultativo. Ela é capaz de utilizar uma grande variedade de fontes fermentáveis de carbono, como frutose e glicose, e fontes não fermentáveis de carbono, como etanol, glicerol, piruvato e lactato (Wills, 1990; Maris *et al*., 2000; Rolland *et al*., 2002).

Vários aspectos metabólicos e fisiológicos da célula estão condicionados à concentração de glicose presente no meio. A curva de crescimento da levedura *S. cerevisiae* (Figura 2) apresenta fases distintas do ponto de vista metabólico e de cinética de crescimento. Após um curto período de adaptação ao meio (fase lag de crescimento), as células iniciam uma divisão celular a cada hora e meia (fase exponencial de crescimento) também chamada de fase logarítmica (LOG), com a energia proveniente da fermentação da glicose (Wills, 1990; Johnston, 1999).

A grande disponibilidade de glicose no meio causa o fenômeno conhecido como repressão catabólica ou “repressão por glicose” (Gancedo, 1998). No metabolismo fermentativo, independentemente da presença ou não de oxigênio (O_2), encontram-se reprimidos os genes envolvidos na síntese de enzimas

necessárias para a utilização de galactose ou dissacarídeos e os genes requeridos na utilização de fontes não fermentáveis de carbono (Fuge e Werner-Washburne, 1997; Maris *et al.,* 2000; Johnston e Kim, 2005; Kaniak *et al*., 2004).

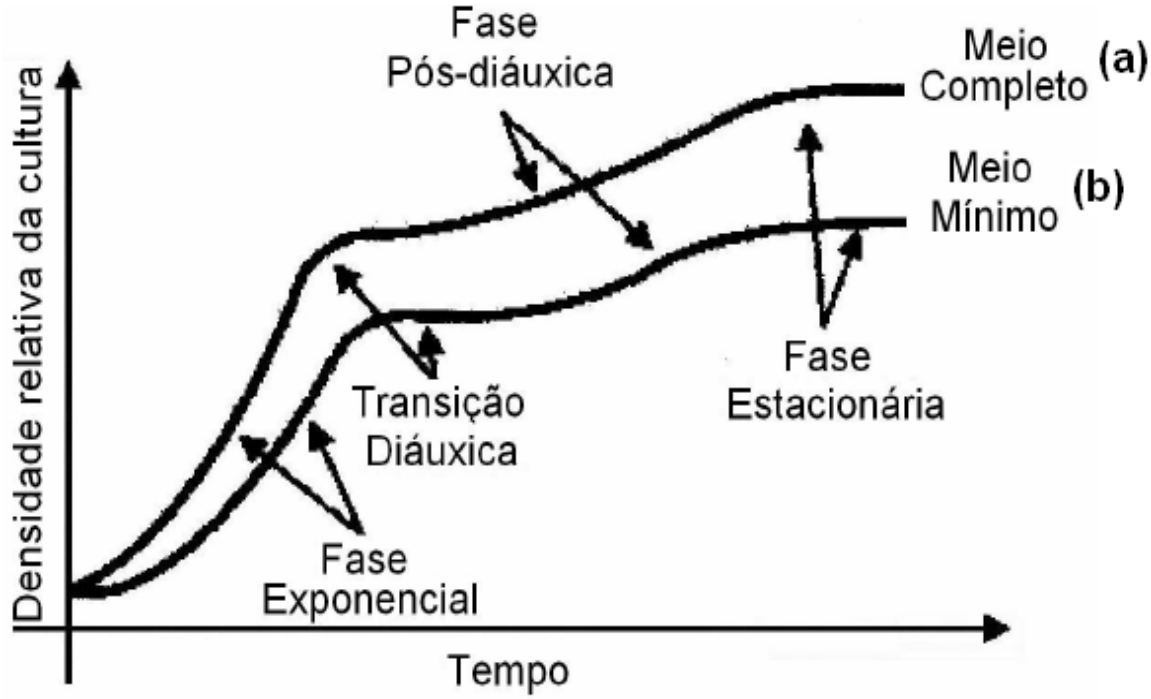


Figura 2. Metabolismo celular de carboidratos da levedura *Saccharomyces cerevisiae* quando crescida em meio completo (a) e meio mínimo (b). (Adaptado de Fuge e Werner-Washburne, 1997).

Quando a concentração de glicose cai abaixo do limite de repressão (menos de 0,2%) ocorre uma reprogramação gênica para que as células possam ser preparadas para o metabolismo respiratório. Essa fase é conhecida como fase diáuxica que é caracterizada por uma desrepressão catabólica dos genes envolvidos na respiração refletida pela indução das enzimas relacionadas com a respiração e dos componentes da cadeia transportadora de elétrons (de Vries e Marres, 1988; Maris *et al*., 2000). As células reassumem a divisão celular em um ritmo mais lento, uma divisão a cada três ou quatro horas, metabolizando o etanol produzido durante a fermentação. Essa fase é chamada de fase pós-diáuxica ou fase estacionária inicial (Fuge e Werner-Washburne, 1997). Quando há um

esgotamento dos nutrientes essenciais, refletido numa alta densidade celular, as células entram na fase estacionária, caracterizada por uma baixa atividade catabólica, onde estas são mais resistentes a uma série de condições de estresse e são capazes de sobreviver à inanição por um longo período (Maris *et al.,* 2000).

1.1.1.2.2. O metabolismo dos ácidos nucléicos e a importância de *RNR4* na levedura *Saccharomyces cerevisiae*

Nos seres vivos, existem dois tipos de ácidos nucléicos: o ácido desoxirribonucléico (DNA) e o ácido ribonucléico (RNA), sendo responsáveis pelo armazenamento e transmissão da informação genética importantes na transmissão dos caracteres genéticos. Os ácidos nucléicos são grandes moléculas constituídas por unidades menores denominadas nucleotídeos. Cada nucleotídeo, por sua vez, é constituído por um ácido fosfórico ligado a uma pentose que se liga a uma base nitrogenada (Snustad e Simmons, 2001).

Para que ocorra manutenção correta da replicação e reparo dos danos produzidos no DNA, é necessária a atividade da enzima tetramérica, ribonucleotídeo redutase (RNR), que é essencial para o metabolismo dos ácidos nucléicos, pois ela catalisa a redução de nucleotídeos difosfato (NDP) para o correspondente desoxinucleotídeo difosfato (dNDP), um essencial passo para a biosíntese de desoxinucleosídeo trifosfato (dNTPs) (Stubbe e Donk, 1998).

A enzima ribonucleotideo redutase (RNR) da levedura *S. cerevisiae* consiste de quatro subunidades diferentes (Figura 3): duas grandes (Y1, Y3) e duas menores (Y2, Y4). A perda de Y4 em mutantes de levedura *S. cerevisiae rnr4Δ* aparentemente pode ser compensada pela super-expressão de Y2 e quando ocorrem danos ao DNA, a subunidade Y3 é altamente induzida (Elledge e Davis, 1987). Estudos mostram que o mutante *rnr4Δ* exibe um crescimento mais lento quando comparado ao selvagem e foi encontrada sensibilidade ao agente físico UVC, à psoralenos foto-ativados (Strauss *et al.,* 2007) e ao agente químico cloreto de estanho ($SnCl_2$) (Basso *et al.,* 2008).

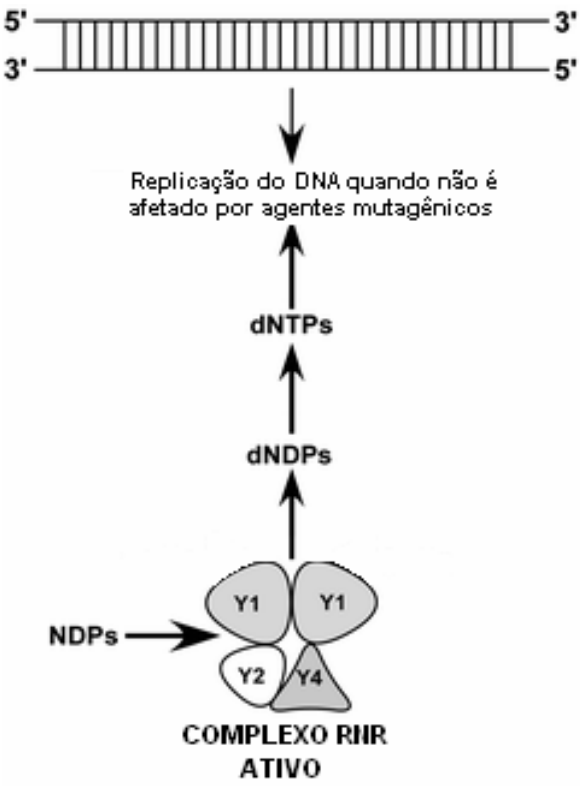


Figura 3. Estrutura da enzima RNR. Na ausência de danos no DNA, o complexo RNR é composto pelo homodímero Y1Y1 e o heterodímero Y2Y4 que converte NDPs a dNDPs em níveis adequados para a replicação do DNA (Adaptado de Strauss *et al*., 2007).

1.1.2. O fungo *Moniliophthora perniciosa*

O fungo *Crinipellis perniciosa* renomeado para *Moniliophthora perniciosa*, (Aime e Phillips-Mora, 2005), trata-se de um basidiomiceto e é o agente causador da doença vassoura-de-bruxa que afeta o cacaueiro (*Theobroma cacao*, Figura 4).

Trata-se de um fitopatógeno bastante eficiente, representando um dos maiores limitantes à produção de cacau na América do Sul e Caribe (Thorold, 1975). O cacau tem uma grande importância sócio-econômica para a maioria dos países, em particular o Brasil, que é o quarto produtor mundial e possui o quinto maior parque industrial chocolateiro do mundo (Dias, 2001).

A doença foi descrita pela primeira vez por Alexandre Rodrigues Ferreira, entre os anos de 1785 e 1787, porém só se tornou conhecida quando os sintomas

ocorridos no Suriname em 1895 foram descritos por Went em 1904 (Griffth *et al.*, 1994).

A provável origem do *M. perniciosa* é a Bacia Amazônica, onde ele ocorre de forma endêmica, sendo que sua introdução na Bahia foi confirmada em 1989 (Pereira *et al*., 1996).

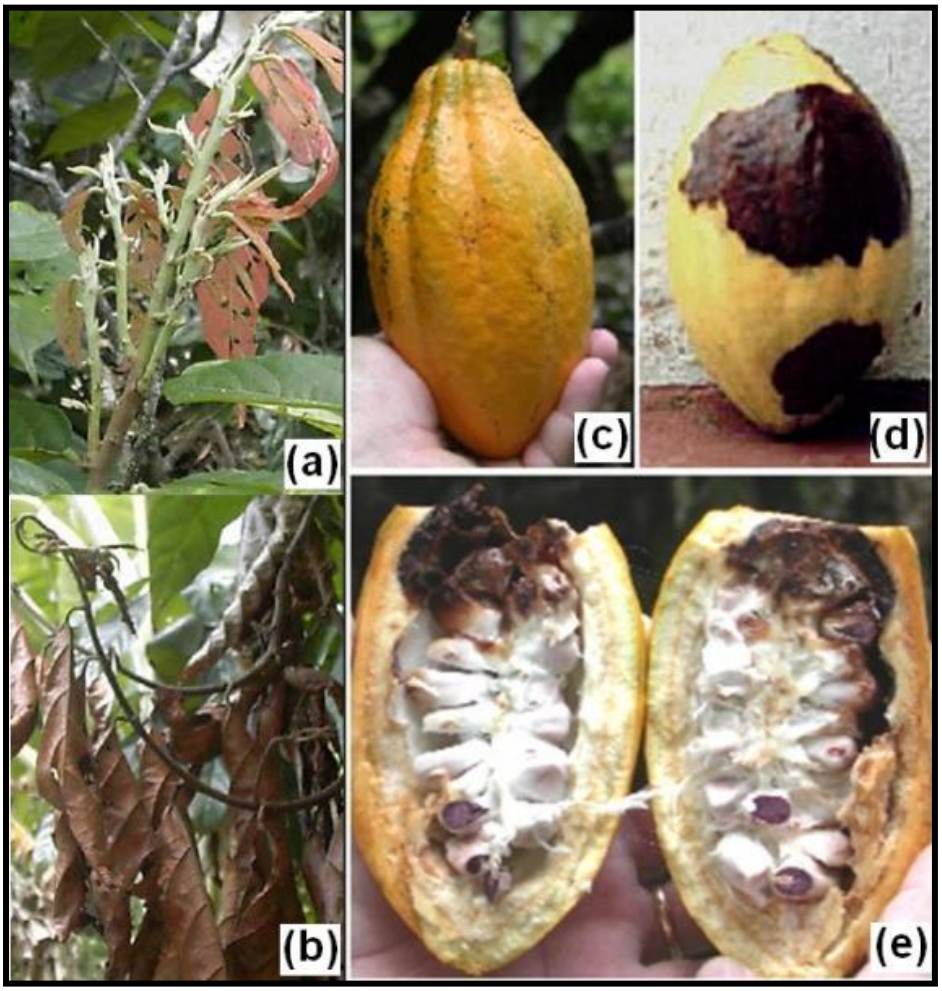


Figura 4. O fungo *Moniliophthora perniciosa* afeta o cacaueiro (*Theobroma cacao*) (a) e os frutos sadios (c) onde após um período de 10 semanas a infecção provoca os sintomas visíveis de vassoura seca no cacaueiro (b) e necrose nos frutos (d) e (e). (Adaptado de Micheli, 2003. (fotos: J. Cascardo)).

Além do cacaueiro, *M. perniciosa* também é capaz de infectar espécies de *Herrania* da família Sterculiaceae (Bastos e Andebrhan, 1986) e tem sido encontrado em associação com várias plantas hospedeiras das famílias Solanaceae (espécies do gênero *Solanum*), Bixacaceae (espécie do gênero *Bixa*

acutifólia) e Malpighiaceae (espécies do gênero *Heteropterys acutiflia*), que não são relacionadas ao cacaueiro (Bastos e Andebrhan, 1986; Resende *et al*., 2000; de Arruda *et al*., 2003a; 2003b).

O controle da doença tem-se baseado na poda fitosanitária, controle químico e biológico e o uso de cultivares resistentes a partir de germoplasmas selvagens (Anderbrhan *et al*., 1995). Entretanto, uma das alternativas mais promissoras para o manejo da vassoura-de-bruxa é a elaboração de novas estratégias de controle através do estudo moleculares (Gesteira *et al*., 2003; Ceita *et al*., 2007), apesar do pouco conhecimento a respeito dos mecanismos bioquímicos, genéticos, fisiológicos e moleculares deste fungo.

O tamanho total do genoma do *M. perniciosa* foi descrito por Rincones *et al*., (2003): possui cerca de 30Mpb, distribuídos em 8 cromossomos, com aproximadamente 8.000 genes. As alterações bioquímicas durante o processo infeccioso da doença foram descritas por Scarpari *et al*. (2005); Meinhardt *et al*. (2006) determinou métodos de crescimento separando as fases dicariótica e monocariótica; testes de avaliação toxicológica foram descritos por Filho *et al*. (2006); e a identificação do fungo por um protocolo de extração de DNA, via amplificação espécies especifico do gene codificando actina, foi recentemente descrito por Melo *et al*. (2007).

1.1.2.1. O ciclo de vida do fungo *Moniliophthora perniciosa*

Trata-se de um fungo que apresenta um ciclo de vida hemibiotrófico (Griffith e Hedger, 1994), apresentando uma fase biotrófica, responsável pelos sintomas de vassoura verde e uma fase necrotrófica, responsáveis pelos sintomas de vassoura seca no cacaueiro (Figura 5). Seu ciclo é comparável em muitos aspectos com de outros basidiomicetos, em que dois núcleos dentro do basídio sofrem cariogamia seguida de meiose e migração dos núcleos aos quatro basidiósporos formados (Delgado,1976; Alexopoulos, 1996).

Os basidiósporos, única forma infectante da doença (Frias *et al*., 1991), germinam formando um micélio primário (monocariótico) entre as células dos tecidos meristemáticos do cacaueiro, como gemas vegetativas, frutos em desenvolvimento, brotos e almofadas florais (Queiroz *et al*., 2003). A resposta do hospedeiro, localizada no ponto de infecção, resulta em um considerável aumento

dos tecidos e no desenvolvimento de ramos laterais, sintomas característicos de hiperplasia e hipertrofia que levam a perda indiretas na produção de cacau (Frias *et al.*, 1991; Orchard *et al.*, 1994; Silva, 1997). Quando há infecção nos frutos, as sementes tornam-se impróprias para o consumo e há também a formação de frutos de coloração escura em forma de cenouras ou morangos (Figura 5) (Silva *et al*., 2002).

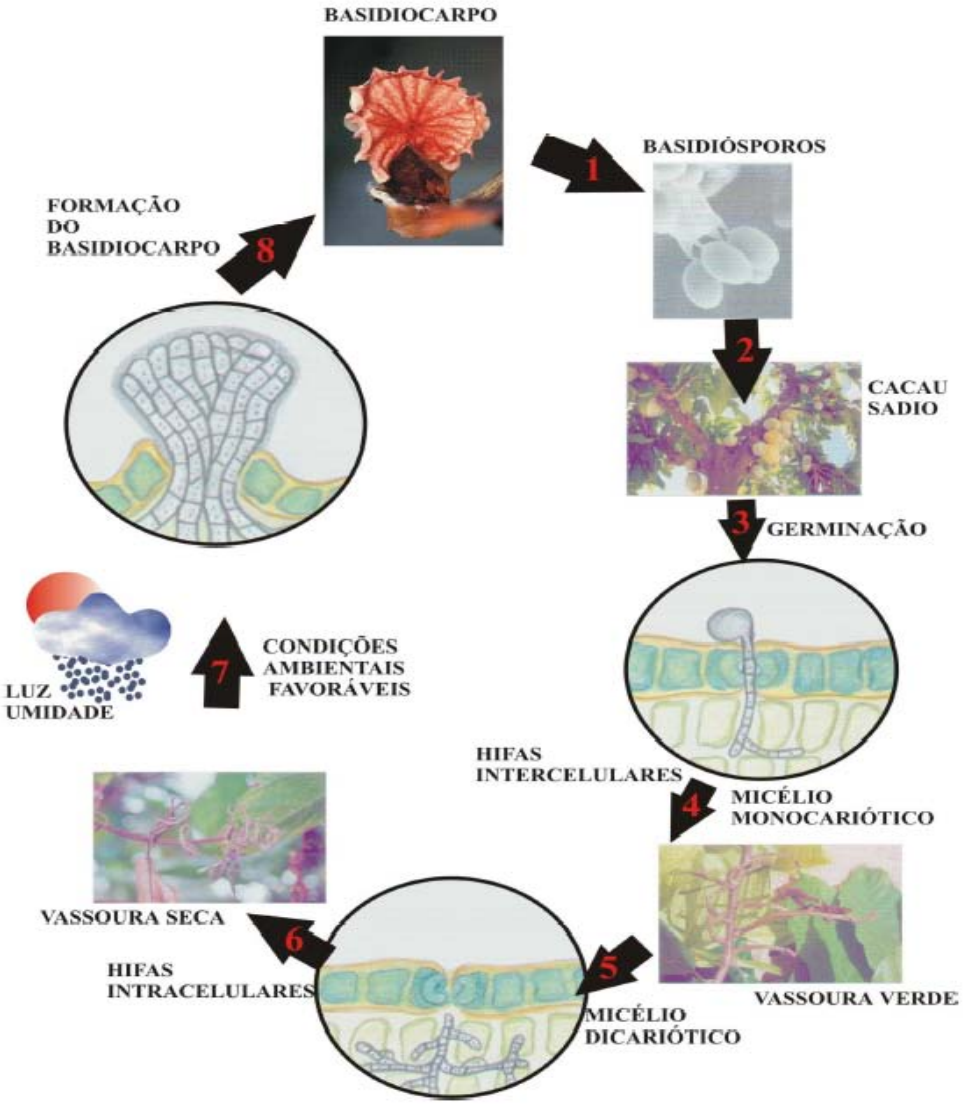


Figura 5. Ciclo de vida do fungo *Moniliophthora perniciosa* (Adaptado de Cotomacci, 2004).

O fungo permanece na fase biotrófica entre 6 e 9 semanas dentro de tecidos em desenvolvimento, até que a vassoura verde inicie um processo de necrose, alteração associada à mudança do fungo para uma fase necrotróica.

Nessa fase ocorre a fusão de hifas, gerando um micélio dicariótico, reconhecível ao microscópio pela formação dos grampos de conexão em suas hifas (Evans 1979; 1980; Calle *et al*., 1982). A morfologia do ramo infectado pelo fungo guarda semelhança com a forma de uma vassoura, surgindo assim o nome popular de vassoura-de-bruxa ou vassoura seca. Essa fase pode permanecer por longos períodos, até mesmo alguns anos (Evans, 1980).

Periodicamente, sob condições ambientais favoráveis de luminosidade e de períodos intermitentes de umidade e seca (Purdy, 1996), o micélio dicariótico sofre uma morfogênese complexa para formar novos corpos de frutificação (basidiocarpos) (Figura 5); destes, emergem os basídios, os quais carregam os basidiósporos que são liberados sob condições ambientais favoráveis, onde a umidade está próxima a de saturação com temperatura variando entre 20 e 30ºC (Rocha e Wheller, 1985). Entretanto, o fato desses basidiósporos requererem uma alta umidade para manter sua viabilidade e de serem suscetíveis à radiação solar UVB, torna bastante improvável que eles possam ser dispersos a um raio superior a 60 km (Frias *et al*., 1991).

1.2. A interferência de mutagênicos no metabolismo celular de fungos

Agentes mutagênicos podem afetar direta ou indiretamente o metabolismo da célula, seja através da toxicidade celular, por exemplo, estresse oxidativo ou através da genotoxicidade, que induz alterações no material genético, sendo mutagênica ou não.

A manutenção da integridade do material genético é fundamental à vida de todos os organismos. Certas substâncias químicas ou físicas podem agir diretamente sobre o material genético das células (DNA), sugerindo capacidade genotóxica. Assim a genotoxicidade estuda, sob o aspecto genético, o que perturba a vida ou induz a morte tanto a nível de célula, como de organismo (Silva *et al*., 2003). Como conseqüência destes efeitos, a mutagênese vem ser o processo pelo qual a mutação é produzida, causando um efeito tóxico que altera especificamente o material genético da célula, provocando uma alteração no DNA ou no cromossomo (Friedberg *et al.,*1995).

1.2.1. O estresse oxidativo

O estresse oxidativo é um termo genérico que designa o estresse associado à detecção, resposta e proteção de células ou de organismos a espécies reativas de oxigênio (ERO). São consideradas ERO quaisquer moléculas contendo oxigênio capazes de gerar radicais ou espécies químicas cujo último orbital quântico possua um elétron desemparelhado. Essas moléculas são coletivamente chamadas de ERO devido a sua grande reatividade, quando comparada ao oxigênio molecular (O_2) (Fang *et al*., 2002).

As ERO são formadas naturalmente no interior da célula, seja nas mitocôndrias ou nos cloroplastos, ou ainda nos peroxissomas, como produtos secundários dos processos de fotossíntese e respiração (Apel e Hirt, 2004); também podem ser sintetizados pela célula para desempenhar funções específicas, como por exemplo, os fagócitos que produzem radicais superóxido para atacar bactérias que tenham sido fagocitadas (Fang *et al*., 2002).

As maiores fontes de ERO endógenas incluem o ânion superóxido ($O_2^{\bullet -}$), o radical hidroxil ($OH^{\bullet}$), o oxigênio *singlet* ($^1O_2$) e o peróxido de hidrogênio (H_2O_2) (Boveris, 1998; Fridovich, 1998; Nickoloff e Hoeckstra, 1998; Dickinson e Schweizer, 1999).

1.2.1.1. Os compostos capazes de gerar estresse oxidativo

O estresse oxidativo pode ter origem endógena, através da fisiologia da própria célula, uma vez que mecanismos, como a cadeia transportadora de elétrons, geram radicais livres; como também pode ser de origem exógena, através da presença de compostos químicos sintéticos, herbicidas ou fungicidas capazes de gerar ERO ou de estratégias de defesa de vegetais ao ataque de patógenos (Landis *et al*., 2005).

1.2.1.1.1. O peróxido de hidrogênio (H_2O_2)

O H_2O_2 não é um radical livre por definição, pois trata-se de uma molécula quimicamente estável. É um intermediário reativo do O_2 que se torna perigoso pelo alcance que possui (se difunde facilmente através de membranas), e por não

reagir imediatamente, pois pode migrar pela célula e atingir alvos distantes do local de sua formação.

É uma ERO importante pela sua capacidade de gerar o radical $OH^{\bullet}$ através sua clivagem por metais de transição, tais como Fe^{2+} e Cu^{2+} pela reação de Fenton, (Fridovich, 1998; Boveris, 1998), e sendo deste modo, um intermediário reativo do oxigênio formado em diversos eventos celulares:

- O H_2O_2 endógeno é formado principalmente na matriz mitocondrial, durante o processo de redução do oxigênio (Fridovich, 1998);
- É um subproduto da assimilação oxidativa de várias fontes de carbono e nitrogênio, por peroxissomos e glioxissomos (Forman e Thomas, 1986);
- É liberado por algumas bactérias, onde este pode atravessar as membranas biológicas e lesionar as células do hospedeiro (Oga, 2003).

1.2.1.1.2. O paraquat (PAQ)

O PAQ (dicloreto de 1,1'-dimetil-4-4'-bipiridina) é um herbicida bastante utilizado na agricultura devido o seu baixo preço comercial e por possuir efeito positivo contra uma grande variedade de ervas daninhas (Ekmekci e Terzioglu, 2005). Apesar de ser considerado relativamente seguro, já existem relatados de efeitos deletérios em células de plantas, bem como em células do pulmão de mamíferos onde se tem atribuído à suas propriedades redox (Kappus e Sies, 1981).

O mecanismo de ação está possivelmente ligado a um ciclo redox, onde a molécula de paraquat é reduzida pela NADPH-citocromo c redutase (Bonneh-Barkay *et al*., 2005). Quando o elétron do PAQ reduzido é passado para o O_2, é produzido radical $O_2^{\bullet -}$ e outros ERO, como o H_2O_2, e OH^{-} (Ayaki *et al*., 2005).

A presença de enzimas celulares protetoras como superóxido dismutase (Sod) e a própria reação de Fenton levam a subsequente produção de H_2O_2 e radical OH^{-} que são altamente reativos. Deste modo, uma única molécula de PAQ pode sofrer repetidos ciclos de redução e oxidação produzindo grandes quantidades de ERO.

Ocorre então um desbalanceamento entre a geração de radicais de oxigênio, aumentada, e a capacidade celular de defesa, diminuída, e por conseqüência, diferentes danos podem ocorrer, como mutações no DNA,

desnaturação de proteínas, peroxidação de lipídios ou danos à clorofila das plantas, podendo levar a perda da integridade da membrana ou ainda à morte celular (Peter *et al*., 1992).

1.2.1.1.3. O cloreto de estanho ($SnCl_2$)

O $SnCl_2$ é um típico exemplo de agente químico capaz de induzir estresse oxidativo nas células. Está presente na constituição de ligas metálicas ou no revestimento de outros metais (protegendo-os da ação da corrosão) (Rüdel, 2003); nas obturações dentárias (Von Mayenburg *et al.,* 1991); no revestimento de latas que acondicionam alimentos e líquidos (de Groot *et al.,* 1973; Ikem e Egiebor, 2005). Dentre outras utilidades o estanho também está presente, em pequenas quantidades, em imitações de jóias (Olivarius *et al.,* 1993).

O estanho (Sn) por ser um elemento químico de ocorrência natural na atmosfera (está presente no ar, água, solos, bem como em plantas e animais que vivem na terra ou nas águas), podendo estar presente nos tecidos humanos (Schroeder *et al.,* 1964; Greger e Baier, 1981). No entanto, não há evidências científicas de que o estanho seja um elemento essencial para o ser humano e que haja uma dose diária recomendada (Johnson e Greger, 1982).

O limite máximo de ingestão para compostos de Sn admitido pelo FDA (Food and Drug Administration) nos EUA é de 300 mg de Sn/kg de alimento seco (Chmielnicka *et al.,* 1981; Burba, 1983). No Reino Unido, esse limite é de 200 mg de Sn/kg de alimento seco e 150 mg de Sn/kg de líquido seco (Blunden e Wallace, 2003). A Associação Brasileira das Indústrias da Alimentação (Abia) preconiza um limite de 0,25 mg de Sn/g de alimento seco (Abia, 1998).

Uma característica de importância biológica desse metal consiste na sua capacidade de formar compostos catiônicos organometálicos de alta solubilidade lipídica, capacitando-os a atravessar membranas biológicas e a exercer seus efeitos tóxicos no interior das células de mamíferos, de humanos e bactérias (McLean *et al*., 1983 a; 1983b; Hamasaki *et al*., 1993).

De acordo com os resultados descritos na literatura em relação à genotoxicidade e à mutagenicidade dos sais de estanho, Olivier e Marzin (1987) constataram o efeito mutagênico para diferentes sais de estanho ($SnCl_2$ e SnF_2) na cepa *Escherichia coli* PQ37; Ashby e Tennant (1991) relataram a atividade

carcinogênica do $SnCl_2$ para a glândula tireóide de ratos; Bernardo-Filho *et al*. (1994) descreveram a indução de funções SOS (Induteste) em cepas de *E.coli* tratadas com diferentes concentrações de $SnCl_2$; Cabral *et al*. (1998) constataram o efeito mutagênico, do $SnCl_2$, observando que este sal gerou transversões, transições e deleções em plasmídeo pAC 189, desta forma, esses autores comprovaram o potencial mutagênico deste íon; Dantas *et al*. (1996); Assis *et al*. (1998a e 1998b); Dantas *et al.* (1999); e de Mattos *et al*. (2000) relataram que o efeito genotóxico deste íon é mediado pela produção de ERO.

Mais recentemente, foi descrito a atividade genotóxica induzida por este composto em levedura *S. cerevisiae* e bactérias (Pungartnik *et al*., 2005; Viau *et al.,* 2006).

1.2.2. Compostos capazes de gerar danos ao DNA

1.2.2.1. A radiação ultravioleta (UV) A luz UV (UVC, 254nm) é um agente mutagênico físico que gera vários fotoprodutos na molécula de DNA. Lesões que ocorrem nas bases pirimidinas são fortemente correlacionadas a fatores de mutagenicidade, ocasionando lesões que interferem no pareamento normal de bases, esse tipo de lesão é chamado de dímero de pirimidina (Friedberg *et al*., 1995).

Dependendo da forma isômera em que esses dímeros se encontram, podem ocorrer distorções na hélice do DNA, o que ocasiona parada da replicação, devido ao impedimento da adição de novas bases. Porém existe uma forma de reparo dessas lesões, esta se encontra na atividade de uma enzima conhecida como fotoliase, que é ativada quando exposta à luz azul/ normal (Friedberg *et al*., 1995).

A radiação UVC também pode levar à formação de pontes proteína–DNA e quebras na cadeia de DNA. Entretanto, a freqüência dessas lesões aumenta com o comprimento de onda da radiação UV (UVB e UVA). Pontes intercadeias DNA–DNA induzidas por UVC correspondem a 0,1 a 1% do numero total de dímeros de pirimidina estimados (Silva *et al*., 2003).

Um outro tipo de lesão provocada pela luz UV são os fotoprodutos 6-4, que é produzida pela ligação entre o carbono 6 de uma timina com o carbono 4 da timina adjacente e o anel ciclobutano, formado pela ligação entre o carbono 4 e o

carbono 5 de duas timinas adjacentes. Essas lesões ocasionam uma grande distorção na dupla hélice do DNA (Lehmann, 2000).

1.2.2.2. Óxido de 4-nitroquinoleína (4NQO)

O composto químico 4NQO é considerado um agente químico UV-mimético, porque ao reagir com as bases púricas, produz adutos que causam importantes alterações na cadeia do DNA (*bulky lesions*), causando quebra nas fitas e lesões do tipo 8-hidroxi-2'-deoxiguanosina (8-OH-dG) (Friedberg *et al*., 1995).

Os estudos acerca deste composto iniciaram-se por volta de 1955, sendo utilizado como uma droga antimalárica, onde foi observada pela primeira vez a sua atividade tumoricida *in vitro* e *in vivo* em astrócitos (Sakai *et al*., 1955). Em 1957, Nakahara e colaboradores, mostraram que o 4NQO era um potente agente carcinogênico, porém a espécie reativa responsável pela ação carcinogênica, (formado durante sua metabolização), somente foi identificada anos depois (Tada e Tada, 1976).

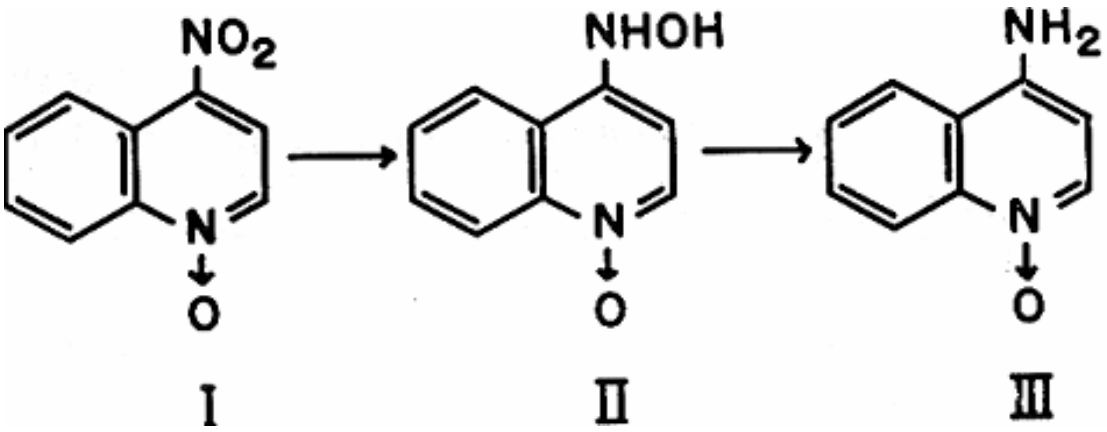


Figura 6. Conversão metabólica do composto 4NQO. (I) óxido de 4-nitriquinoleína; (II) óxido de 4-hidroxiaminoquinolina; (III) óxido de 4-aminoquinolina (Adaptdo de Sugimura *et al*., 1966).

Inicialmente, o 4NQO é convertido a óxido de 4-hidroxiaminoquinolina (4HAQO) e depois a óxido de 4-aminoquinolina (4HQO) o qual é um aceptor final de hidrogênio (Figura 6), que interage com C-8 de guanina, e também com os nitrogênios exocíclicos N2 da guanina e N6 da adenina, gerando adutos. Desta forma, o 4NQO é um pró-mutagenico, cuja atividade carcinogênica é dependente do ciclo redox da célula (Silva *et al*., 2003).

A conversão de 4NQO a 4HQO causa danos à célula porque o 4HQO, quando ativado via ciclo redox, é uma espécie reativa de nitrogênio que libera, principalmente o $O_2^{\bullet -}$, também gerando ERO. O 4NQO, por si só, é inativo como aceptor de hidrogênio. Para que ocorra a conversão do 4NQO a 4HAQO funcionam como doadores de hidrogênio $NADH_2$ e $NADPH_2$ (Sugimura *et al*., 1966).

1.2.3. Sistemas de detecção de genotoxicidade

A detecção de genotoxicidade se dá por dois eventos: pela mutação à frente, "*forward mutation"*, ocorrendo uma mudança de um fenótipo selvagem para um fenótipo mutante, e pela mutação reversa, "*reverse mutation*", quando após ter ocorrido uma mutação (fenótipo mutante), o gene pode mutar novamente, produzindo cópias normais (fenótipo selvagem) (Friedberg *et al.,* 1995).

Existem diversos testes utilizados para se detectar efeitos genotóxicos, como por exemplo, o teste de *Salmonella*/ microssomo, anteriormente denominado Teste de AMES (Maron e Ames, 1983) e o sistema de detecção de seleção de mutantes via sistema de canavanina (Gocke e Manney, 1979).

1.2.3.1. A canavanina

A L-canavanina (2-amino-4-(guanidinoxi) ácido butírico) (Figura 7) é um aminoácido não protéico, presente nos feijões, nas cebolas, em sementes, na alfafa, e outras plantas superiores, sendo uma forma análoga natural da L-arginina, possuindo um importante papel de defesa contra insetos e herbivoria e estocagem de nitrogênio (Rosenthal, 1977; 2001).

A canavanina funciona como um potente antagonista que exibe atividade anti-metabólica em muitos sistemas *in* vivo e *in vitro* (Akaogi *et al*., 2006).

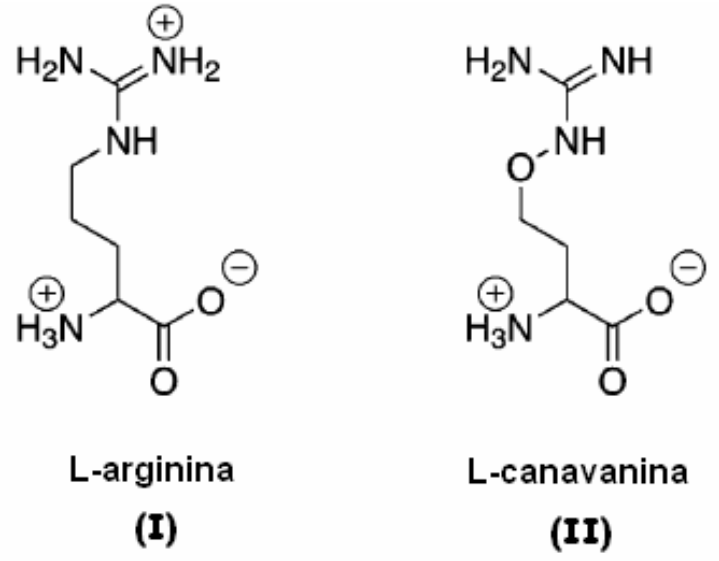


Figura 7. Estrutura molecular da L-arginina (I) e de seu análogo, L-canavanina (II) (Adaptado de Luzzi e Marletta, 2005).

O ensaio para a detecção de indução de mutação com a canavanina mede a resistência dos compostos tóxicos causados por ela. O gene *CAN1*$^{+}$, quando ativado, promove a importação através da arginina permease de L-arginina e de seu análogo tóxico L-canavanina para o interior celular (Rodriguez *et al*., 2007) e sendo ativada pela RNA arginil transferase (Rosenthal, 1977). Uma mutação à frente no gene que codifica para a arginina permease, inibe a importação de canavanina, impedindo o acúmulo de canavanina nas células o que exibe o fenótipo de resistência à canavanina (Rodriguez *et al*., 2007).

2. Objetivos

2.1. Objetivo geral

O objetivo do presente trabalho foi a realização da análise comparativa de sensibilidade e mutabilidade à agentes mutagênicos ou que gerem estresse oxidativo nos fungos *Saccharomyces cerevisiae* e *Moniliophthora perniciosa*.

2.2. Objetivos específicos

- Determinar a influência da enzima ribonucleotideo redutase no mecanismo de ação do $SnCl_2$ em *S. cerevisiae*.

- Determinar a influência da ploidia na sensibilidade à mutagênicos e no processo autofágico em *M. perniciosa*.

- Obter mutantes de *M. perniciosa* no gene *CAN1* com métodos já desenvolvidos para *S. cerevisiae*, com o propósito de obter uma marca de seleção para *M. perniciosa*.

3. Capítulo I

4. Capítulo II

Ploidy affects macroautophagy and resistance of *Moniliophthora perniciosa* to mutagens

Basso, T.S.; Melo, S.C.O.; Pungartnik, C.; Cascardo, J.C.M.; Brendel, M*.

Dept. Ciências Biológicas – Universidade Estadual de Santa Cruz (UESC), Rodovia Ilhéus Itabuna, Km 16, Salobrinho, Ilhéus, Bahia, Brazil (42665000)

(to be submitted to Fungal Genetics and Biology)

* corresponding author: martinbrendel@yahoo.com.br

ABSTRACT

The hemibiotrophic basidiomycete *Moniliophtora perniciosa* is responsible for the "witches' broom disease" in cacao (*Theobroma cacao)*. In order to understand why *M*. *perniciosa* changes from monokaryotic to dikaryotic stage, and whether this change goes along with altered sensitivity/resistance to genotoxins, we decided to compare sensitivities of both types of *M*. *perniciosa* cultures and of basidiospores to different mutagens. Cells grown for 7 days on liquid glycerol or glucose medium, were submitted to fragmentation, and 1mL of the monokaryotic/dikaryotic broken-hyphae and basidiospores were exposed to four different mutagens, the chemicals H_2O_2, Paraquat and 4NQO and radiation with UVC. The resistance ranking to the oxidative stress-inducing mutagens H_2O_2 and PAQ was: basidiospores > monokaryon (glycerol) > dikaryon (glycerol) > dikaryon (glucose) whereas the directly DNA damaging agents UVC and 4NQO (elcited the same resistance response in basidiospores and mono/dikaryotic cells glycerol-grown) and only glucose-grown dikaryotic cells were significantly more sensitive. Molecular analyses of gene expression of Mp*ATG8* (autophagy related gene) of dikaryotic mycelia grown either in glucose or glycerol treated with H_2O_2 or 4NQO shows that glucose induces autophagic process mediated by Mp*ATG8* while glycerol represses this process. Apparently, growth in glucose does not only accelerate hyphal growth but also leads to higher sensitivity to all four tested mutagens.

Key words: *Moniliophtora perniciosa*, genotoxicity, ploidy effects, catabolic response, autophagic process

Abbreviations: Mp, *Moniliophthora perniciosa*; UVC, UV_{254nm} radiation; 4NQO, 4-nitroquinoline-1-oxide; PAQ, Paraquat

INTRODUCTION

While most eukaryotic organisms exist as diploids, with two sets of gametic genomes residing in the same nucleus, most basidiomycete fungi exist as dikaryons in which the two genomes are separate nuclei that are physically paired and that divide in a coordinated manner during hyphal extension (Clark and Anderson, 2004). The basidiomycetes constitute a large fungal group encompassing many diverse forms including the rusts and smuts that cause plant disease, the mushrooms and other large forms such as boletes, puffballs, and bracket fungi, and also the yeast-like ones, such as *Cryptococcus neoformans*, which is an opportunistic pathogen that causes meningitis in immunocompromised humans (Casselton and Olesnicky, 1998).

The basidiomycete fungi derive their name from the fact that meiosis occurs in specialized cells, called basidia, and the resulting spores, the basidiospores, are produced outside the cell. This is in contrast to the other major group of fungi, the ascomycetes, where meiosis occurs in a cell called the ascus and the resulting ascospores develop inside the cell (Casselton and Olesnicky, 1998).

Filamentous microorganisms, such as many fungi, grow as physically structured colonies in which the distance among growing tips is correlated with relatedness by descent. In these structured systems, cell division and growth occur predominantly at the hyphal tips near the periphery of the colony and dispersal is restricted such that the descendants remain in adjacent areas. In a filamentous colony, the cells in the interior of the colony may be dormant or may replicate only infrequently (Clark and Anderson, 2004). A characteristic feature of fungal hyphae is the presence of a large number of nuclei in a common cytoplasmic environment (Maheshwari, 2005). Once two compatible nuclei are present within the hyphal tip cell, all subsequent growth is in the form of a mycelial dikaryon rather than as a diploid. This prolonged dikaryotic phase is a characteristic of basidiomycete fungi and can be maintained indefinitely (Casselton and Olesnicky, 1998) making it the predominant stage of the basidiomycete life cycle (Clark and Anderson, 2004).

Witches' broom disease (WBD) of cacao, caused by the hemibiotrophic basidiomycete *Moniliophthora perniciosa*, exhibits a succession of symptoms that are

caused during the biotrophic phase of the fungus in the plant. WBD begins when wind-borne monokaryotic spores infect young meristematic tissues through stomatal openings and form intercellular monokaryotic hyphae. After 2 to 3 months of infection, the fungus produces clamp connections and undergoes dikaryotization, marking the transformation to necrotrophic phase characterized by intracellular growth (Calle *et al.,* 1982).

The fungus may also be using a wider variety of nutrient sources from the plant during necrotrofic growth, including relatively non-readily fermentable polymer substrates and fatty acids from host membranes (Goodwin *et al*., 2001). Changes in host nutrient availability to the pathogen have previously been observed in this interaction for host sulfur compounds, which appear to be more accessible during the necrotrophic than the biotrophic phase (Goodwin *et al.,* 2001).

In vitro M. perniciosa phase of growth is dependent on the nutrient source. Only glycerol is required to maintain the monokaryotic mycelia, while the dikaryotic phase of growth is kept in any carbon source (Meinhardt *et al.,* 2005). The mycelium of *M. perniciosa* in necrotrophic phase either *in vivo* or *in vitro* is a heterokaryon with two nuclei per cell. The process of nuclear division and cytokinesis of the tip cell is complex, involving development of a clamp connection, formation of two septa, and fusion of the clamp with the penultimate cell, so that a nucleus of each type is compartmentalized into a new tip cell and the penultimate cell, respectively. This process has been observed and follows the usual sequence of nucleus fission and migration for dikaryotic basidiomycetes (Sheperd *et al*., 1993).

In order to understand why *M*. *perniciosa* changes from the monokaryotic to the dikaryotic stage, and whether this change goes along with altered sensitivity/resistance to genotoxins, we decided to compare sensitivity to different mutagens of both types of *M*. *perniciosa* cultures.

MATERIALS and METHODS

Growth conditions. *M. perniciosa* hyphae were grown without shaking at 25ºC in complete media: dikaryotic cells were always pre-grown on CPD (2% glucose, 2% bacto-peptone); monokaryotic and basidiospores on CPG (2% glycerol, 2% bacto-peptone); 2% agar was added for solid media. Species specificity of *M. perniciosa* cultures was confirmed by PCR amplification of the actin gene (Melo *et al*., 2006).

Exposure of *M. perniciosa* to mutagens and cell survival. Sensitivity of *M. perniciosa* broken hyphae to different mutagens was determined as described by Filho *et al*. (2006) on solid media (CPD/CPG) supplemented either with 4NQO (2 to 8 μM), or PAQ (100 to 400 μM), or H_2O_2 (1 to 4 mM). Briefly, 1 mL of *M. perniciosa* broken hyphae suspension was applied to three plates of CPD or CPG containing each mutagen, and the absolute number of ‘‘pseudo-colonies’’ was counted after 7 days of incubation at 25ºC. Each of the three plates gave rise to a number of ‘‘pseudo-colonies’’/mL of suspension. Control plates without mutagen yielded *M. perniciosa* colonies for determination of 100% survival. UVC treatment was performed by irradiation of *M. perniciosa*–inoculated plates (CPD/CPG) with exposure doses between 0 and 96 J/m^2 (Spectrolinker, Spectronics Corp., Westburg, NY). Results are expressed as the percentage of survival related to the untreated controls. Results are the mean of at least three independent experiments, and the error bars represent standard deviation as calculated by the GraphPad Prism® program (GraphPad Software Inc., San Diego, CA).

Basidiospore mutagen treatment. One hundred microliters of a suspension containing about 10^5/mL of *M. perniciosa* basidiospores, were spread onto CPG agar plates that were supplemented with two antibiotics (streptomycin and chloramphenicol at a concentration of 180 and 150 mg/mL, respectively). Treated plates with each mutagen (same doses as above) were incubated at 25ºC for 7 days and then screened for appearance of *M. perniciosa* mini-colonies (2–5 mm, irregular shape). Inhibition of germination tube formation was observed after 4.5 h of

incubation with each mutagen in light microscope. Results are the mean of at least three independent experiments, and the error bars represent standard deviation as calculated by GraphPad Prism® program.

Primer design. Primers were designed based on genome sequences of *M. perniciosa* amplified from a cDNA libray constructed by A.B. Leal from differentiated mycelia grown in artificial media for basidiocarp production (The Genome Project, http://www.lge.ibi.unicamp.br/vassoura). The chosen sequence is homologous to *ATG8* gene and thus was named Mp*ATG8*. Its primers are: Forward gATgAgCACCCCTTTgAgAA; Reverse ATAgACgAATTggCCCACAg. GenBank® accession number EU477413 (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

Gene expression: Extraction of mRNA and further handling of the samples was as described by Melo *et al*. (2006). Briefly, samples were collected after following incubation either in CPD or CPG for 7 days and later added either 4-NQO (20 µM) or H_2O_2 (10 mM) to be collected after 30 min, 1h and 2h of growth with or without new media. RNAs were isolated with RnaPure kit (QIAGEN®). Analysis of RNA extraction efficiency and gene expression was carried out in 1% agarose gel (PROMEGA®). RNA was purified from DNA by DNase treatment (FERMENTAS®). The first-strand cDNAs were synthesized by the following protocol: 6 µL of reverse transcription reaction mixture containing total cellular RNA, 5 µM of oligo (dT), 5 X RT buffer (260 mM Tris-HCl, 200 mM KCl, 25 mM $MgCl_2$, 2.5% Tween 20), 10 mM dNTPs, and 20 U/mL of RNase inhibitor were incubated at 37°C for 5 min. After adding 200 units of Reverse Transcriptase (Invitrogen®) tubes were incubated at 42°C for 60 min, heated to 70°C for 10 min and then quick-chilled on ice. The cDNA was then used for amplification the samples by PCR. Non-inducible Mp*ACT1* gene was used as internal standard of gene expression. PCR was performed in 25 µL reaction volumes containing 20 ng cDNA, 1.25 µL dNTPs (2.5 mM), 1 µL $MgCl_2$ (50 mM), 2.5 µL of buffer PCR 10X (Tris-HCl 10 mM, KCl 50 mM, $MgCl_2$ 1.5 mM, pH 8.3), 0.2 µM of each primer pair and 0.2 µL of *Taq* DNA polymerase (5 u/µL) (Invitrogen®) and distilled water to complete. Cycling conditions in a thermocycler (Eppendorf MasterCycler®)

consisted of an initial denaturation step at 95^{o}C for 3 min, followed by 30 cycles of 95^{o}C for 50 s, annealing temperature of the primer for 50 s, and 72ºC for 1 min. PCR was completed by a final extension of 7 min at 72ºC. Products were resolved in 1% agarose gel and were visualized with ethidium bromide and ultraviolet illumination. Images were taken and stored by using the Kodak-EDAS® system.

RESULTS and DISCUSSION

Slow-growing *M. perniciosa* cultures are frequently infected by other fungi. We therefore regularly controlled the identity of *M. perniciosa* and verified its monokatyoric (Fig. 1A) and dikaryotic state (Fig. 1B) by microscopic observation of clamp connections, typical for the dikaryotic phase of fungal growth. We also regularly controlled the identity of *M. perniciosa* culture by the highly species-specific PCR-amplification of actin gene (Fig. 1C, Melo *et al*., 2006).

When basidiospores are exposed on agar for 7 days to the mutagens the formation of colonies depends on the influence of the mutagen on tube formation and later on the survival of the growing mycelium. Indeed, Fig. 2 shows that there is no correlation between germination and tube formation and the ability of colony formation of hyphae (Fig. 3), e.g. PAQ inhibited 25% of basidiospore tube formation (highest dose) but allowed only 8% of survival after 7 days of chronic mutagen exposure.

Clearly, monokaryotic broken hyphae, as their dikaryotic counterparts, and basidiospores all responded in an exposure dose-dependent manner to either chronic treatment with chemical mutagens (Fig. 3A, B and D) or to irradiation with UVC (Fig. 3C). However, monokaryotic cells and basidiospores exhibited a significantly higher resistance to all 4 mutagens than their dikaryotic counterparts when grown in glucose (Filho *et al*., 2006). The resistance ranking to the oxidative stress mutagens (H_2O_2, PAQ) was basidiospores > monokaryon (glycerol) > dikaryon (glycerol) > dikaryon (glucose) whereas the DNA damage agents (UVC, 4NQO) elicited the same resistance response for basidiospores and glycerol-grown mono/dikaryon and only glucose-grown dikaryon weres significantly more sensitive.

Dikaryotic *M. perniciosa* shows an acquired resistance dependent on the carbon source (glucose or glycerol), which is however, not as pronounced as that of monokaryotic cells (Fig. 3); general acquired resistance to the same mutagens when dikaryotic cells were pre-grown in glycerol or were shifted from glucose to glycerol media had also been observed (Santos *et al.,* 2008). This could be explained either by an oxidative stress response to the non-fermentable carbon source glycerol and

the expression of anti-ROS defense genes like Mp*CTT1* and Mp*CTA1* that showed carbon source-dependent regulation, resembling carbon-catabolite repression, or by the enhanced induction of specific ABC transporters (Santos *et al.,* 2008).

The nuclei cooperate, compete or combat. It is proposed that in addition to their classical role in heredity, supernumerary nuclei in filamentous fungi serve as storehouse for nitrogen and phosphorus in the form of DNA to be utilized via degradation by regulated autophagy during unavailability of macronutrients. The recycled breakdown products enable hyphal tips to persistently extend and to forage in new areas (Maheshwari, 2005). Olsson (2001) has described this variable structure as a functional mycelium unit (FMU), a single organism, where nutrient capture and use involves balancing exploratory (hyphal extension) and exploitive (cytoplasm synthesis) modes of growth which can be correlated to the “single cell” response of the “pseudo-hyphae” of *M. perniciosa* (Filho *et al*., 2006).

A molecular analysis of medium-induced variations on expression of Mp*ATG8* in drug treated dikaryotic cells was performed via RT-PCR using the non-inducible actin gene (Mp*ACT1*) as an internal control. We could successfully amplify cDNA of the putative Mp*ATG8* that is involved in autophagy (Nakatogawa et al., 2007). Mp*ATG8* gene yielded a different pattern of expression indicating that the carbon source had not only a significant influence on cellular sensitivity/resistance to different mutagens but also altered intra-cellular nutrient turnover. While Mp*ATG8* is repressed in the presence of non-fermentable carbon source, e.g. glycerol during H_2O_2 (Fig. 4A) and 4NQO (Fig. 4B) exposure, it is induced in the presence of glucose (Fig.4A and B). Apparently, absence of glycerol is a signal for imminent shortage of nutrients. This could trigger expression of genes controlling the autophagic process, especially after damage (Fig. 3A to D) because a higher rate of recycling is necessary to remove possible damaged proteins.

Unidirectional transfer of nutrients from plant host to pathogen represents a most revealing aspect of the parasitic lifestyle of plant pathogens. Wei *et al*. (2004) provided evidence that glycerol played a significant role in nutrient transfer from infected plants to the fungal pathogen *Colletotrichum gloeosporioides* f. sp. *malvae*. In addition, the sucrose non-fermenting–related protein kinase from *C. gloeosporoides* f.

sp. *malvae*, which is overexpressed during penetration *in planta*, was found to have higher expression when the fungus was grown in glycerol (Goodwin and Chen, 2002). Also aquaglycerolporins have been identified in several plant species capable of facilitating the efflux of glycerol to the apoplast (Zardoya *et al*., 2002). Glycerol in the apoplast can, therefore, be readily taken up by the pathogen.

An earlier study showed that the non-melanized fungal cell wall is permeable to glycerol diffusion (de Jong *et al*., 1997). Unlike sugars, which require elaborate membrane transport systems, glycerol, as an uncharged compound with only three carbon atoms, can penetrate the fungal membrane by simple diffusion (Gancedo *et al*., 1968). It is known that plant tissue at the peripheral zone of fungal infection sites has reduced glycerol content (Wei *et al*., 2004). Taken together, all of the evidence suggests that glycerol, and possibly lipids, could play a significant role in altering the fungal response to oxidative stress during *in planta* growth, as it can be seen for the *in vitro* response to the mutagens.

Glycerol can be utilized as a sole carbon and energy source by both bacteria and fungi and has been characterized as a significant a metabolite existing in plant cells at a concentration of 1–2 *μ*mol/g fresh weight (Gerber *et al*., 1988), and thus it is possible that glycerol also plays a role in the successful infection of *T. cacao* by *M. perniciosa*. The levels of glycerol used to germinate *M. perniciosa* basidiospores and to maintain the biotrophic phase *in vitro* are comparable with those observed by Scarpari *et al*. (2005), who found significantly higher levels of glycerol in infected green broom tissue of *T. cacao* as compared with uninfected tissues. Indeed, at this stage of infection, monokaryotic cells readily can use the glycerol as an energy source, and therefore autophagic process to recycle nutrients is not needed (Fig. 4A e B, glycerol media); although its catabolism may generate ROS, glycerol also induces ROS defenses (Ames *et al*., 1993) which leads to enhanced resistance to ROS (Fig. 3A and B). High levels of glycerol *in planta,* therefore, may maintain the biotrophic phase during the development of WBD and contribute to enhanced stress resistance of cells.

Once these higher levels of glycerol found in the infected tissues are completely gone in dry broom tissues, the necrotrophic phase of *M. perniciosa*

prevails (Calle *et al*., 1982) and autophagy may now be necessary to obtain nutrients by recycling cellular material from non-growing hyphae (Fig. 4A and B glucose media). Thus absence of glycerol may signal future shortage of nutrients and that may induce dikaryotization of the fungus *in planta* as a decisive step to complete the sexual cycle which leads to basidiocarp and basidiospore formation. Since necrotrophic plant tissue is no longer able to express plant defense mechanisms (e.g. oxidative burst), there is no need for higher ROS protection of the fungus, and therefore the dikaryotic fungus shows higher sensitivity to mutagens (Fig. 3 A to D), Filho *et al*. (2006) and Santos *et al*. (2008). This process occurs in the dry broom where hyphae are already subjected to low contents of readily available nutrients. In this scenario, autophagy could be a parallel process in the fungus to obtain nutrients by digestion of not needed hyphal material in order to progress in the sexual cycle (Fig. 4).

In conclusion, the relative mutagen sensitivity of cells in the dikaryotic state of *M. perniciosa* as compared to their monokaryotic counterparts is most probably caused by the fact that dikaryotic hyphae grow in a dry broom, i.e. in dead/dying plant tissue. Here, the chances of an oxidative attack are much lower, hence high expression of genes expressing anti-ROS defenses is no longer needed. Obviously, a different set of genes is induced that enables the fungus to derive nutrients from other sources, i.e. substances of the dry broom made available via enzymatic degradation (fungal proteases, nucleases or chitinases), and also by an enhanced process of autophagy, where the fungus uses monokaryotic and resting dikaryotic hyphae as a source of nutrients.

ACKNOWLEDGMENTS

Basidiospores were provided by Lívia Santana from Laboratory of Genetic and Molecular Biology - UESC. Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Master Food Inc. (MARS/USA) and Fundação de Amparo a Pesquisa do Estado da Bahia (FAPESB). T.S.B. and S.C.S.M. held FAPESB fellowships and are Master and Doctoral students in Genetics Post-Graduation Program of UESC. M.B. is Visiting Scientist supported by FAPESB.

REFERENCES

Ames BN, Shigenaga MK, Hagen TM. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the United States of America 90:7915-7922.

Calle H, Cook AA, Fernando SY. 1982. Histology of witches´ broom caused in cacao by *Crinipellis perniciosa*. Phytopathology 72:1479-1481.

Casselton LA, Olesnicky NS. 1998. Molecular genetics of mating recognition in basidiomycete fungi. Microbiology and Molecular Biology Reviews 62:55-70.

Clark TA, Anderson JB. 2004. Dikaryons of the basidiomycete fungus *Schizophyllum commune*: Evolution in Long-Term Culture. Genetics 167:1663-1675.

de Jong JC, McCormack BJ, Smirnoff N, Talbot NJ. 1997. Glycerol generates turgor in rice blast. *Nature* 389:244-245.

Filho DF, Pungartnik, Cascardo JCM, Brendel M. 2006. Broken hyphae of the basidiomycete *Crinipellis perniciosa* allow quantitative assay of toxicity. Current Microbiology 52:407-412.

Gancedo C, Gancedo JM, Sols A. 1968. Glycerol metabolism in yeasts. Pathways of utilization and production. European Journal Biochemistry 5:165-172.

Gerber DW, Byerrum RU, Gee RW, Tobert NE. 1988. Glycerol concentrations in crop plants. Plant Science 56:31-38.

Goodwin PH, Chen GY. 2002. Expression of a glycogen synthase protein kinase homolog from *Colletotrichum gloeosporioides* f.sp. malvae during infection of *Malva pusilla*. Canadian Journal Microbiology 48:1035-1039.

Goodwin PH, Li J, Jin S. 2001. A catalase gene of *Colletotrichum gloeosporioides* f. sp. malvae is highly expressed during the necrotrophic phase of infection of round-leaved mallow, *Malva pusilla*. FEMS Microbiology Letters 202:103-107.

Maheshwari R. 2005. Nuclear behavior in fungal hyphae. FEMS Microbiology Letters 249:7-14.

Nakatogawa H, Ichimura Y, Ohsumi Y. 2007. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. Cell 130:165-78.

Meinhardt L, Bellato C, Rincones J, Azevedo R, Cascardo JCM, Pereira GAG. 2006. *In vitro* production of biotrophic-like cultures of *Crinipellis perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*. Current Microbiology 52:191-196.

Melo SC, Pungartnik C, Cascardo JC, Brendel M, 2006. Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete *Crinipellis perniciosa.* General Molecular Research 5:851-855.

Olsson S. 2001. Colonial growth of fungi. In: Howard, RJ, Gow, NAR (Eds.) The Mycota. VIII. Biology of the Fungal Cell. Springer-Verlag, Heidelberg, 125-141p.

Santos RX, Melo SCO, Cascardo JCM, Brendel M, Pungartnik C. 2008. Carbon source-dependent variation of acquired mutagen resistance of *Moniliophthora perniciosa*: similarities in natural and artificial systems. Fungal Genetics and Biology. Accepted.

Scarpari LM, Meinhardt LW, Mazzafera P, Pomella AW, Schiavinato MA, Cascardo JC, Pereira GA. 2005. Biochemical changes during the development of witches'

broom: the most important disease of cocoa in Brazil caused by *Crinipellis perniciosa*. Journal of Experimental Botany 56:865-877.

Shepherd VA, Orlovich DA, Ashford AE. 1993. Cell-to-cell transport via motile tubules in growing hyphae of a fungus. Journal of Cell Science 105:1173-1178.

Wei Y, Shen W, Dauk M, Wang F, Selvaraj G, Zou J. 2004. Targeted gene disruption of glycerol-3-phosphate gehydrogenase in *Colletotrichum gloeosporioides* reveals evidence that glycerol is a significant transferred nutrient from host plant to fungal pathogen. The Journal of Biological Chemistry 279:429-435.

Zardoya R, Ding X, Kitagawa Y, Chrispeels M. 2002. Origin of plant glycerol transporters by horizontal gene transfer and functional recruitment. Proceedings of the National Academy of Sciences of the United States of America 99:14893-14896.

FIGURE LEGENDS

Figure 1 – Photos of clamps (arrows) and no clamps of *M. perniciosa* monokaryotic (A) and dikaryotic (B) mycelia. PCR amplification of species-specific actin gene (C) (line1 – monokaryotic mycelia; line 2 – dikaryotic mycelia).

Figure 2 – Percent spore germination of *M. perniciosa*. 0) control, no mutagen treatment; 1) first dose; 2) second dose; 3) third dose of mutagen.

Figure 3 – Survival of *M. perniciosa* to four mutagens: A) H_2O_2; B) PAQ; C) UVC; D) 4NQO. Dikaryotic broken hyphae grown on glucose (■) or glycerol(▼); monokaryotic broken hyphae (●); and basidiospores(○) grown on glycerol.

Figure 4 – *M. perniciosa* RT-PCR of selected genes in glucose or glycerol medium after A) H_2O_2 B) 4NQO exposure. Numbers between lanes correspond to exposure time (h). Conditions as described in material and methods.

Figure 1

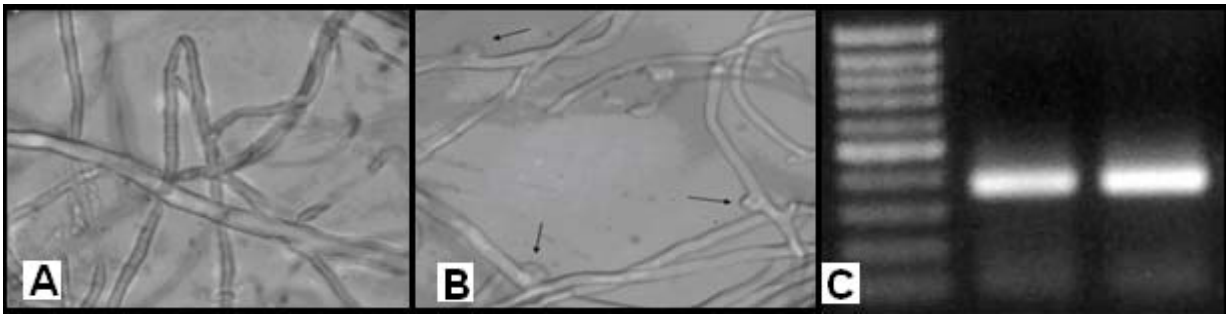


Figure 2

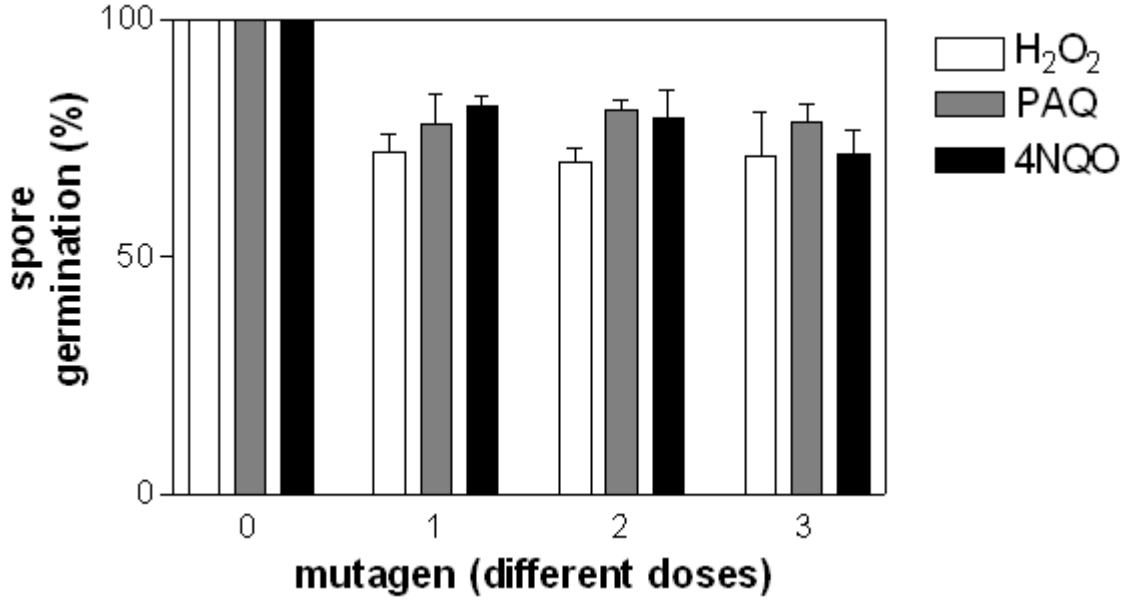

100
50
0
spore germination (%)
0
1
2
3
mutagen (different doses)
H2O2
PAQ
4NQO

Figure 3

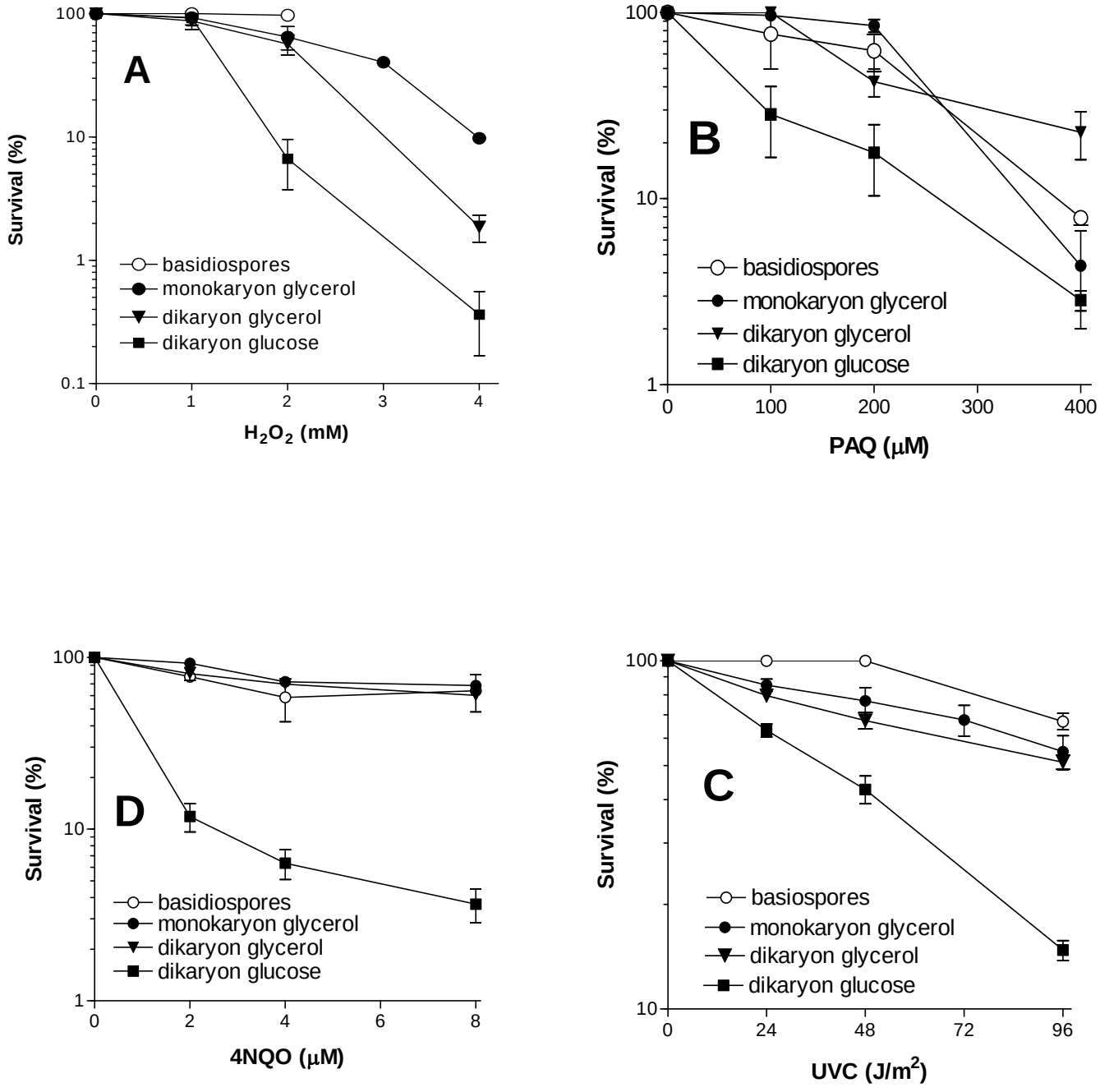
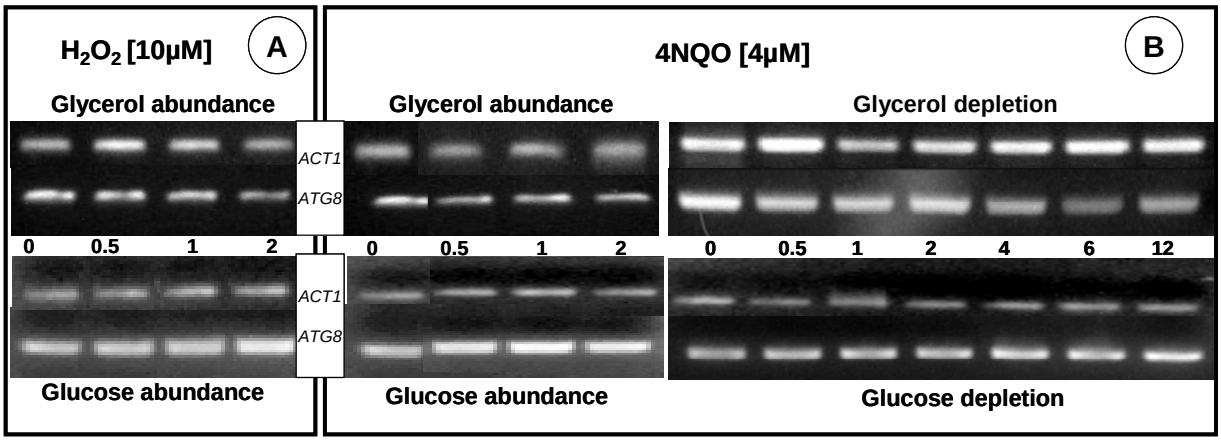

A
Survival (%)
100
10
1
0.1
0
1
2
3
4
H2O2 (mM)
basidiospores
monokaryon glycerol
dikaryon glycerol
dikaryon glucose
B
Survival (%)
100
10
1
0
100
200
300
400
PAQ (μM)
basidiospores
monokaryon glycerol
dikaryon glycerol
dikaryon glucose
D
Survival (%)
100
10
1
0
2
4
6
8
4NQO (μM)
basidiospores
monokaryon glycerol
dikaryon glycerol
dikaryon glucose
C
Survival (%)
100
10
0
24
48
72
96
UVC (J/m2)
basiospores
monokaryon glycerol
dikaryon glycerol
dikaryon glucose

Figure 4


H_2O_2 [10µM]
A
Glycerol abundance
ACT1
ATG8
0 0.5 1 2
ACT1
ATG8
Glucose abundance
4NQO [4µM]
B
Glycerol abundance
Glycerol depletion
0 0.5 1 2
0 0.5 1 2 4 6 12
Glucose abundance
Glucose depletion

5. Capítulo III

Isolation and phenotypic characterization of an arginine permease mutant (*Mpcan1*^{R}) of *Moniliophthora perniciosa*

Basso, T.S.; Pungartnik, C.; Brendel, M.*

Dept. Ciências Biológicas – Universidade Estadual de Santa Cruz (UESC), Rodovia Ilhéus Itabuna, km 16, Salobrinho, Ilhéus, Bahia, Brasil (42665-000)

(in preparation)

* Corresponding author: martinbrendel@yahoo.com.br

ABSTRACT

The basidiomycete fungus *Moniliophthora perniciosa* causes Witches' Broom disease in *Theobroma cacao*. Though its genome has been exhaustively sequenced and several cDNA libraries are available little is known about the fungus' response to mutagens and mutants are not available. Mutagenic studies and the isolation of specific mutants in this multicellular fungus require the treatment of basidiospores, its only single cell and haploid life form. We tested the feasibility of basidiospore mutagenesis in isolating mutant clones with a forward mutational system that is well-described in the single cell fungus *Saccharomyces cerevisiae.* The arginine permease system is useful for mutational studies because all selected canavanine (analog of arginine) resistant mutations are thought to occur at a single locus, *CAN1,* the structural gene for arginine permease. We have isolated *M. perniciosa* canavanine-resistant mutants as a tool to investigate basic genetic events in this hemibiotrophic basidiomycete. The 3 isolated putative Mpcan1^{R} mutants were resistant to 60 mg/mL canavanine A, while the WT type was not. Further phenotypic characterization of the *Mpcan1*-1^{R} - mutant showed that it had unchanged WT-like resistance to UVC treatment but that it was significantly more resistant to H_2O_2 when compared to the WT.

Key words: *Moniliophthora perniciosa*, mutation, arginine permease, canavanine resistance

Abbreviations: Mp, *Moniliophthora perniciosa*; UVC, UV_{254nm} radiation; H_2O_2, hydrogen peroxide; WT, wild type

INTRODUCTION

The basidiomycete *Moniliophthora perniciosa* (formerly *Crinipellis perniciosa*) is the causal agent of witches' broom disease in cacao (*Theobroma cacao*) which is the main factor limiting cacao production in the Americas (Griffith, 2004). A first step in studying this fungus was the sequencing of its genome (http://www.lge.ibi.unicamp.br/vassoura; Rincones *et al*., 2003). With this information homologs to genes of other fungi were discovered and their expression studied (Melo *et al*., 2006; Santos *et al*., 2008). Some genetic tools like transformation, gene replacement and synthetic lethal studies are still lacking or are very cumbersome (Lima *et al*., 2003). In an approach to overcome some of these difficulties we set out to develop an easy transformation system that may function on complementation of auxotrophies, a method generally used in bacteria and yeasts.

Mutational studies in the locus coding for arginine permease (*CAN1*) are promising in *M. perniciosa* because this forward mutation system offers easy selection of only mutational events. Any malfunction of the mutated *CAN1* gene (*can1* mutants) will yield cells devoid of functional arginine permease, so that import of arginine and its homolog L-canavanine are inhibited. Thus, *can1* mutant alleles render the cell resistant to the cell poison L-canavanine so that they, still endowed with the genes for endogenous *de novo* synthesis of arginine, can grow in the presence of L-canavanine while the WT with a functional arginine permease takes up L-canavanine and kills itself (Gocke and Manney, 1979; Whelan *et al*., 1979) Thus, all L-canavanine resistant mutations (allele designation $can1^R$) occur at a single locus.

L-canavanine, a non-protein amino acid present in various beans, clover, onions, seeds and sprouts of alfalfa, and other higher plants, is a natural homologue of L-arginine and enters the cell via the arginine-specific permease Can1p (Rosenthal, 1977; 2001). L-canavanine works as a potent antagonist that exhibits anti-metabolic activity in many living systems *in vitro* and *in vivo*. L-canavanine can compete with L-arginine when cellular enzymes such as arginyl tRNA synthetase, inducible nitric oxide synthase (iNOS), and arginase target free arginine. More importantly, L-canavanine is a substrate for arginyl tRNA

synthetase; L-canavanine can be charged by arginyl tRNA synthetase and replace L-arginine during protein synthesis, creating aberrant canavanyl proteins (Rosenthal, 1977). Although the pKa of the guanidiooxy group of L-canavanine is far less than the value of L-arginine, up to 30% of arginine residues can be replaced by L-canavanine based on analysis of proteins synthesized *in vitro* (Rosenthal, 1977; 2001). This substitution can occur in any arginine-containing protein and results in the production of structurally aberrant, canavanyl proteins (Rosenthal, 1977) as L-canavanine substitutions disrupt the tertiary and/or quaternary structure that is responsible for the unique three dimensional conformation of a protein (Rosenthal et al., 1989). This results in lower or abolished enzymatic activity (Rosenthal, 1977; 2001) and a potentially rapid degradation of the malformed proteins (Knowles *et al*., 1975). And thus, persistent presence of intracellular L-canavanine may result in apoptotic cell death.

Induced mutagenesis and isolation of mutants is facilitated in haploid organisms or life forms of diploids as a gene defect is not complemented by a WT allele. Thus, in a multi-cellular basidiomycete fungus as *M. perniciosa* basidiospores are best suited for this task. Once mutated and growing, the may change to mono- and later to dikaryotic multi-cellular hyphae that all contain the same mutant allele of the *CAN1* gene and therefore all display the phenotype resistance to L-canavanine. Broken hyphae of these mono- or dikaryotic mutants can then be manipulated for phenotypical characterization, transformation assays and studies of heterologous gene expression.

We used low-dose UVC as mutagenic agent to induce mutations in the *CAN1* locus and both UVC and oxidative stress-generating hydrogen peroxide for testing the stability of the isolated *M. perniciosa* can1^{R} mutants and possible collateral phenotypic changes.

MATERIALS and METHODS

Growth conditions. *M. perniciosa* hyphae were grown without shaking at 25ºC in complete media: basidiospores on CPG (2% glycerol, 2% peptone); dikaryotic cells were always pre-grown on CPD (2% glucose, 2% peptone); 2% agar was added for solid media.

Basidiospore treatment. One hundred microliters of a suspension containing about 10^5/mL of *M. perniciosa* basidiospores, was spread on canavanine minimal medium plates (2% glycerol, 2% YNB, 2% agar and 30 or 60 mg/mL L-canavanine sulfate, 180 mg/mL streptomycin and 150 mg/mL chloramphenicol), and, after drying the plates in sterile air, irradiated with a single dose of UVC (150 J/m^2), and incubated at 25^{o}C for 15 days in the dark.

Growth conditions of *M. perniciosa* and selection of mutants. Colonies formed by the germination of mutagenized basidiospores on media containing either 30 or 60 mg/mL L-canavanine (putative can1 mutants) were transferred by replica plating to CPD to allow further growth and dikaryotization. The putative can1^R mutants were once more tested for L-canavanine resistance (replica plating on L-canavanine media 30 and 60 mg/mL): isolated hyphae from the agar-grown colonies were subjected to glass-bead vortexing in order to break the hyphae (Filho *et al*., 2006). Broken hyphae were grown at 25°C for 7 days in liquid culture without shaking and, after vigorous vortexing, plated on CPD media and UVC-irradiated (24, 48, 72, 96 J/m^2) or chronically exposed to hydrogen peroxide (broken hyphae plated on CPD medium containing 1, 2, 3, 4 mM H_2O_2). Survival of broken hyphae and formation of colonies was recorded after 7 days of incubation at 25°C. Results are the mean of at least three independent experiments, and the error bars represent standard deviation as calculated by GraphPad Prism® program.

RESULTS and DISCUSSION

Three putative *Mpcan1*^{R} mutants were selected which were resistant to L-canavanine (up to 60 mg/mL – Fig. 1) as compared to the sensitive wild type. One mutant *Mpcan1-1*^{R}, was further phenotypically characterized and was found to have unaltered, WT-like resistance to UVC treatment, as expected (Fig. 2). However, this mutant was significantly more resistant to H_2O_2 when compared to the WT (Fig. 3).

The successful UVC-induced mutagenesis in *M. perniciosa* revealed that the Mp*CAN1* gene, responsible for the uptake of arginine and L-canavanine, has a similar mutational response as previously shown for the yeast *S. cerevisiae* (Gocke and Manney, 1979). This mutational system can now be studied in more detail, e.g., by using more potent chemical mutagens, like ethyl methanesulfonate (EMS) that has been shown to be an excellent mutagen, proving a high rate of induced mutations at low cytotoxicity (van Zeeland *et al*., 1983). With most of the mutagenized cells surviving treatment by EMS, the mutant yield is much higher than when using UVC, as initially used by us. A final proof that indeed the malfunction of Mp*CAN1* gene is responsible for resistance to L-canavanine is still lacking, but may be provided by successful heterologous complementation with *S. cerevisiae CAN1* gene of *Mpcan1-1*^{R} mutants. This is necessary because recent studies of L-canavanine resistance in microorganisms have revealed that other genes may influence resistance to this arginine analog as well.

In mutagenized yeast *S. cerevisiae*, the expression of resistance to L-canavanine, is strongly dependent on the metabolic state of the cell. Frequency of mutations recovered after UVC exposure or to X-rays varies with culture conditions. Apparently the frequency of recovered mutants is determined by three factors: (i) the potential mutants still possess enough permease activity to take up some of the cell poison, and therefore some are killed before they can express the mutant genotype. This sensitivity depends strongly on the endogenous free arginine, which is in turn influenced by the growth medium; (ii) the rapid decay of existing permease molecules and the inability of the potential mutants to re-synthesize this protein results in a rapidly increasing change of expression when selection for canavanine resistance is delayed; (iii) during the time of decay of

permease activity, repair of the mutagen-induced DNA damage appears to occur (Gocke and Manney, 1978).

Whelan *et al*. (1979) developed a system of strains and growth media to allow efficient detection of forward mutation, reversion, complementation, and suppression at the canavanine-resistance *(CAN1*) locus of *S. cerevisiae.* Allelic complementation was not observed, despite testing of a large number of allele pairs, and alleles suppressible by the ochre suppressor *SUP11* were absent from a sample of 48 spontaneous mutants and occurred infrequently (7%) among a sample of UVC-induced mutants. Infrequent mutant types included L-canavanine-resistant mutants capable of arginine uptake and alleles thought to represent deletions or inversions. The recovery of spontaneous canavanine-resistant mutants is reduced dramatically in a strain of *S. cerevisiae* that carries a suppressed *can1*-100 allele and is permeable to, and auxotrophic for, thymidylate. This effect does not occur in an isogenic strain that neither takes up nor requires the nucleotide. However, it is observed for another isogenic strain which is permeable to but not auxotrophic for thymidylate, indicating that the effect is related to thymidylate permeability. Enhanced uptake of L-canavanine in these cells seems to be responsible for their increased sensitivity and this growth inhibition accounts for the diminished mutant recovery. This suggests that the elevated transport of L-canavanine in the thymidylate auxotroph is unlikely to be due to enhanced suppression of the *can1*-100 allele or to activation of the yeast general amino acid permease (Kohalmi and Kunz, 1989).

In naturally resistant bacterial isolates, the mechanism of resistance appears to be decomposition of L-canavanine to a non-toxic product. However, in a resistant mutant derived from a naturally susceptible isolate, the mechanism of resistance was an impaired uptake for L-canavanine. The toxic effect of L-canavanine in *Cryptococcus* results from the incorporation of L-canavanine into the protein component that is essential for the synthesis of proteins and RNAs (Polacheck and Kwon-Chung, 1986).

Resistance to L-canavanine can also be modulated via regulation of the expression of the arginine permease. Btn2p interacts biochemically and functionally with Rsg1p, a down-regulator of the Can1p arginine and lysine permease (Chattopadhyay and Pearce, 2002). Rsg1p localizes to a distinct structure toward the cell periphery, and strains lacking Btn2p (*btn2*Δ mutants) fail

to correctly localize Rsg1p. Both *btn2*Δ and *rsg1*Δ mutants are sensitive to the arginine analogue L-canavanine. Furthermore, *btn2*Δ strains, like *rsg1*Δ strains, demonstrate an elevated rate of uptake of [14C]-arginine, which shows these mutants to have increased intracellular levels of arginine. Overexpression of *BTN2*, on the other hand, results in a decreased rate of arginine uptake. These results indicate that altered levels of Btn2p can modulate arginine import (and therefore also uptake of L-canavanine) through localization of the Can1p-arginine permease regulatory protein, Rsg1p. Btn1p is a vacuolar/lysosomal membrane protein, and *btn1*Δ mutants suppress both the canavanine sensitivity and the elevated rate of uptake of arginine displayed by *btn2*Δ *rsg1*Δ strains. Up-regulation of *BTN2* expression in *btn1*Δ strains may facilitate either a direct or indirect effect on intracellular arginine levels (Chattopadhyay and Pearce, 2002).

The nascent polypeptide-associated complex (NAC) is an abundant and phylogenetically conserved protein complex. It is composed of two subunits and interacts with nascent polypeptide chains emerging from the ribosome. It seems to protect the nascent polypeptide chains from premature interaction with other cell proteins, but also associates with DNA junctions and is involved in other processes including transcription regulation and mitochondrial protein import. In fission yeast *Schizosaccharomyces pombe* NAC is associated with ribosomes but a significant fraction remains in a free form. The NAC alpha subunit contains an ubiquitin-associated (UBA) domain, which is found in several proteins involved in the ubiquitin-proteasome pathway for protein degradation. However, NAC does not associate with ubiquitin chains and mutants lacking NAC did not exhibit any obvious defects in protein degradation. NAC seems responsible for the natural level of L-canavanine resistance which allows the hypothesis that NAC is involved in protein quality control (Andersen *et al*., 2007). Based on the possible physiological role previously observed, and knowing that H_2O_2 oxidizes proteins, we can speculate that our *Mpcan1-1*^{R} is most probably a mutant in the arginina permease gene. A modification on permeability of membranes in *M. perniciosa* could be a cellular signal to induce gene expression alterations. NAC protein was found amongst 6 proteins in a proteomic analyses of salt stress responsive proteins in rice root (Yan *et al.,* 2005). NAC complex and other genes (e.g. superoxide dismutase or catalases) could be induced in the *Mpcan1-1*^{R} mutant and hence contribute to hyperesistance to H_2O_2.

Despite all these complications to clearly associate resistance to L-canavanine to a defect in the *CAN1* locus, this system of selectable forward mutations has proven very useful in initiation mutational studies in *M. perniciosa*. Once all mutational procedures are optimized for *M. perniciosa* using this relatively simple and economic mutagenesis assay, we will proceed with the isolation of mutant alleles of the *URA3* gene, encoding dihydroorate decarboxylase, the enzyme catalyzing an important step in the synthesis of uracil (Boeke *et al*., 1986). Once we possess strains of *M. perniciosa* with stable *ura3* mutant alleles we can study the heterologous expression of yeast *URA3* gene in this basidiomycete fungus. When successful, a whole array of *URA3*-containing yeast/*E.coli* shuttle vectors can be used for improvement of the transformation procedures for *M. perniciosa* and for construction of *M. perniciosa*/yeast or *M. perniciosa/ E. coli* shuttle vectors.

ACKNOWLEDGEMENTS

Basidiospores were provided by Lívia Santana from Laboratory of Genetic and Molecular Biology - UESC. Research supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Master Food Inc. (MARS/USA) and Fundação de Amparo a Pesquisa do Estado da Bahia (FAPESB). T.S.B. held a FAPESB fellowship and is a Master student in Genetics Post-Graduation Program of UESC. M.B. is Visiting Scientist supported by FAPESB.

REFERENCES

Andersen M, Sample CA, Hartmann-Petersen R. 2007. Characterization of the nascent polypeptide-associated complex in fission yeast. Molecular Biology Reports 34:275-281.

Boeke JD, LaCroute F, Fink GR. 1986. A positive selection for mutants lacking orotidine-5´-phosphate decarboxylase activity in yeast: 5´-fluoro-orotic acid resistance. Molecular General Genetics 197:345-346.

Chattopadhyay S, Pearce DA. 2002. Interaction with Btn2p is required for localization of Rsglp: Btn2p-mediated changes in arginine uptake in *Saccharomyces cerevisiae.* Eukaryotic Cell 1:606-612.

Filho DF, Pungartnik C, Cascardo JCM, Brendel M. 2006. Broken hyphae of the basidiomycete *Crinipellis perniciosa* allow quantitative assay of toxicity. Current Microbiology 52:407-412.

Gocke E, Manney TR. 1979. Expression of radiation-induced mutations at the arginine permease (*CAN1*) locus in *Saccharomyces cerevisiae*. Genetics 91:53-66.

Griffith GW. 2004. Witches´ brooms and frosty pods. Biologist 51:71-75.

Kohalmi SE, Kunz BA. 1989. Enhanced canavanine uptake is associated with nucleotide permeability in a thymidylate auxotroph of *Saccharomyces cerevisiae*. Current Genetics 15:129-134.

Knowles SE, Gunn JM, Hanson RW, Ballard FJ. 1975. Increased degradation rates of protein synthesized in hepatoma cells in the presence of amino acid analogues. Biochemical Journal 146:595-600.

Lima JO, dos Santos JK, Pereira JF, de Resende ML, de Araújo EF, de Queiroz MV. 2003. Development of a transformation system for *Crinipellis perniciosa*, the causal agent of witches' broom in cocoa plants. Current Genetics 42:236-240.

Melo SCO, Pungartnik C, Cascardo JCM, Brendel M. 2006. Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete *Crinipellis perniciosa*. Genetics and Molecular Research 5: 851-855.

Polacheck I, Kwon-Chung KJ. 1986. Canavanine resistance in *Cryptococcus neoformans*. Antimicrobial Agents and Chemotherapy 29:468-473.

Rincones J, Meinhardt LW, Vidal BC, Pereira GA. 2003. Electrophoretic karyotype analysis of *Crinipellis perniciosa*, the causal agent of witches broom disease of *Theobroma cacao*. Mycological Research 107:452-458.

Rosenthal GA. 2001. L-canavanine: a higher plant insecticidal allelochemical. Amino Acids 21:319-330.

Rosenthal GA, Reichhart JM, Hoffmann JA. 1989. L-canavanine incorporation into vitellogenin and macromolecular conformation. Journal of Biological Chemistry 264:13693-13696.

Rosenthal GA. 1977. The biological effects and mode of action of L-canavanine, a structural analogue of L-arginine. Quarterly Review of Biology 52:155-178.

Santos RX, Melo SCO, Cascardo JCM, Brendel M, Pungartnik C. 2008. Carbon source-dependent variation of acquired mutagen resistance of *Moniliophthora perniciosa*: similarities in natural and artificial systems. Fungal Genetics and Biology. Accepted.

van Zeeland AA, Mohn GR, Aaron CS, Glickman BW, Brendel M, de Serres FJ, Hung CY, Brockman HE.1983. Molecular dosimetry of the chemical mutagen ethyl methanesulfonate. Quantitative comparison of the mutagenic potency in *Neurospora crassa* and *Saccharomyces cerevisiae*. Mutation Research 119:45-54.

Whelan WL, Gocke E, Manney TR. 1979. The *CAN1* locus of *Saccharomyces cerevisiae*: fine-structure analysis and forward mutation rates. Genetics 91:35-51.

Yan S, Tang Z, Su W, Sun W. 2005. Proteomics analysis of salta stress-responsive proteins in rice root. Proteomics 5:235-44.

FIGURE LEGENDS

Figure 1 – Putative *Mpcan1*^{R} mutants selected which resistant to L-canavanine (up to 60 mg/mL).

Figure 2 – Sensitivity to UVC exposure (broken hyphae grown for 7 days in the dark) of WT and putative *Mpcan1-1*^{R} mutant. WT (filled squares); putative *Mpcan1-1*^{R} mutant (open squares).

Figure 3 – Sensitivity to H_2O_2 exposure (broken hyphae grown for 7 days and exposed to H_2O_2, 1, 2, 3, 4 mM) of WT and putative *Mpcan1-1*^{R} mutant. WT (filled squares); putative *Mpcan1-1*^{R} mutant (open squares).

FIGURE 1

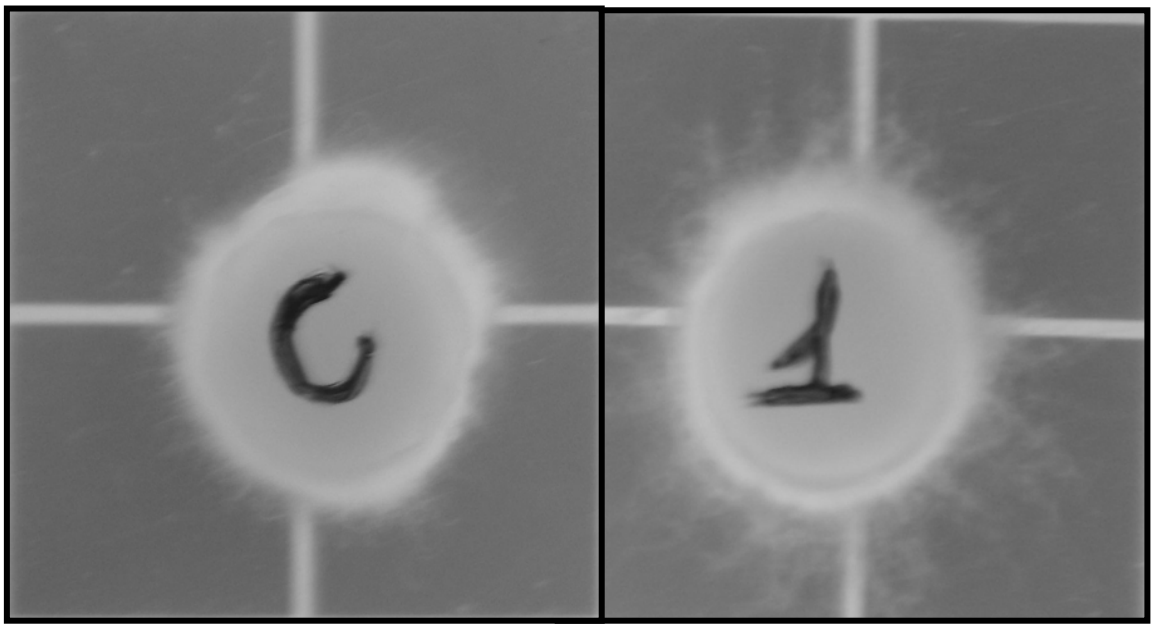


FIGURE 2

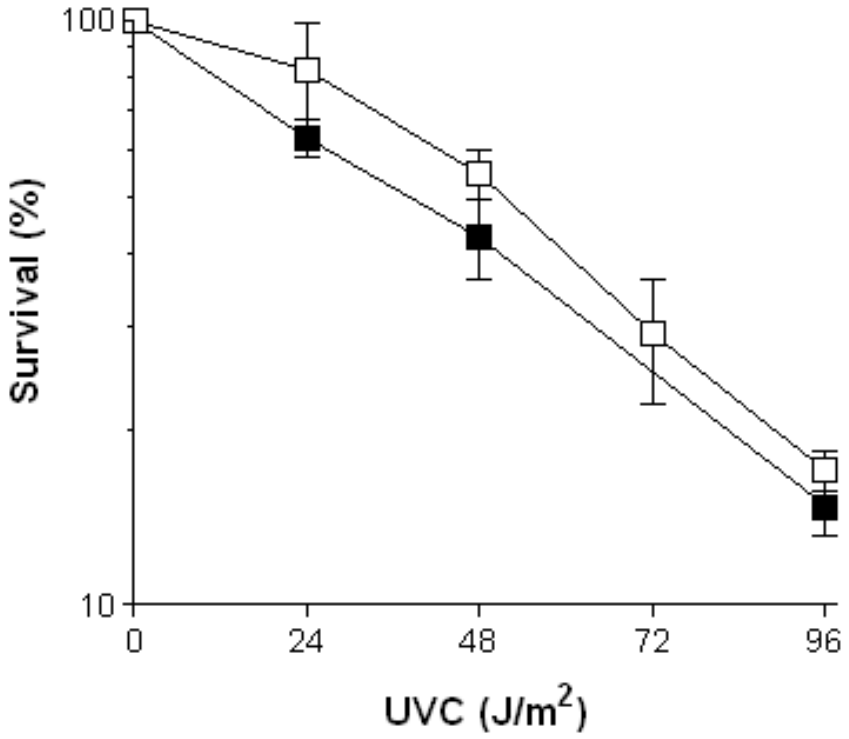
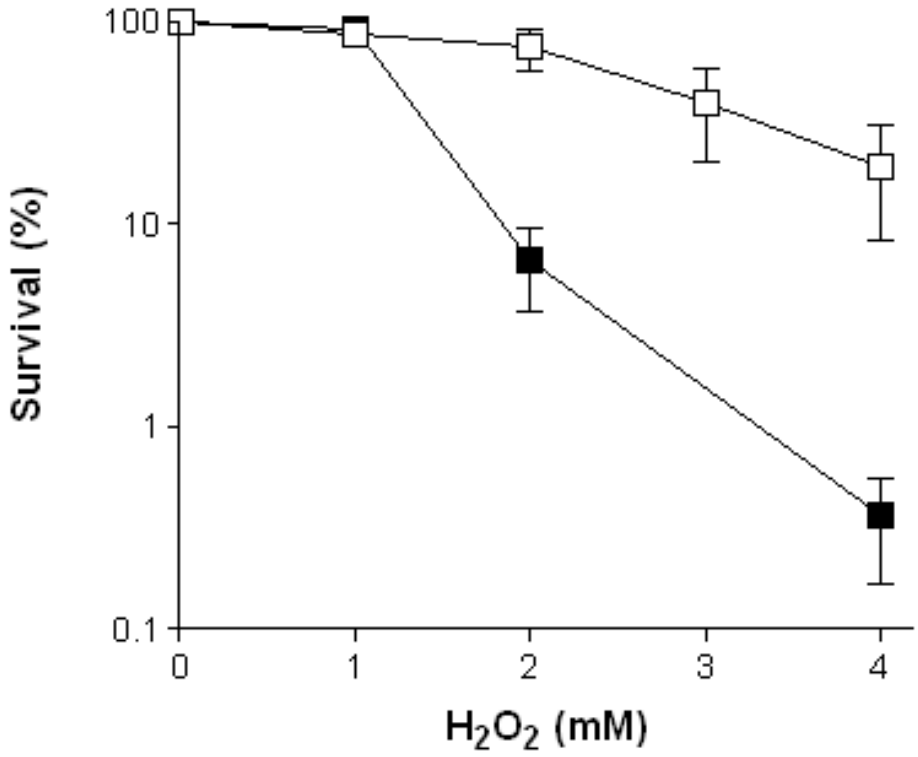


FIGURE 3



6. Discussão

6.1. Mutantes *rnr4Δ* de levedura *Saccharomyces cerevisiae* e sua sensibilidade ao $SnCl_2$

Quatro genes (*RNR1* a *RNR4)* codificam para as quatro diferentes proteínas da enzima tetramérica ribonucleotideo redutase responsável pela produção de deoxirribonucleotídeos necessários para a síntese de DNA. Sua regulação está ligada ao ciclo celular e induzida por danos provocados no DNA de organismos como *E. coli*, *S. cerevisiae* e *H. sapiens* (Elledge *et al*., 1993).

No presente estudo foi avaliado a sensibilidade ao $SnCl_2$ de um mutante deficiente na produção de proteína Rnr4 na levedura *S. cerevisiae*. Pelo fato desta se tratar de um organismo anaeróbico facultativo, as células crescidas em diferentes fases de crescimento, logarítmica (LOG) ou estacionária (STAT), são comumente utilizadas para se testar a sensibilidade do agente químico $SnCl_2$ em seu metabolismo geral (Viau *et al*., 2006).

Uma cultura de levedura em fase STAT, possui uma maior resistência intrínseca à maioria dos agentes genotóxicos, uma vez que ocorre uma mudança no seu metabolismo com relação a fatores de estresse, entre eles importação de metais, ERO endógena ou exógena; por outro lado, as células em LOG interagem fortemente com o ambiente através da ativação de permeases da importação e exportação, como alguns transportadores do tipo ABC (Decottignies *et al*., 1998). Assim, as células em LOG são muito mais sensíveis aos metais tóxicos, através do transporte ativo de um íon metálico exógeno, como o Sn^{2+}, que interfere negativamente com a homeostase metálica, que por sua vez, pode causar graves distúrbios metabólicos, levando à morte celular (Viau C, comunicação pessoal).

O fato do mutante *rnr4Δ* não chegar verdadeiramente à fase STAT, deixa-o em um tipo de fase LOG constante, tornando-o mais sensíveis à ação genotóxica do $SnCl_2$ (Figura 2/ Capítulo I). Isto seria explicado pela incapacidade de concluir mitose/ citocinese após os 5 dias de crescimento em cultura líquida, onde estaria associada a alguns passos metabólicos típicos da fase LOG.

Strauss *et al*. (2007) demonstrou que o mutante *rnr4Δ* apresenta sempre maiores índices de brotos que o WT. O menor processamento da enzima RNR no mutante *rnr4Δ* (Y1Y3Y2Y2) é devido à substituição da subunidade pequena Y4 no

heterodímero Y2Y4 pelo Y2 no homodímero Y2Y2, o que deixa a célula com significativa baixa na produção normal de *pools* de dNTP (Figura 3/ Introdução e Revisão Bibliográfica) (Perlstein *et al*., 2005). Essa baixa na produção de *pools* de dNTP leva a um lento crescimento celular e aumento significativo da sensibilidade ao agente físico UVC aparentemente por não permitir um reparo eficiente dos danos provocados ao DNA no mutante *rnr4Δ* (Strauss *et al*., 2007).

A sensibilidade observada do mutante *rnr4Δ* ao $SnCl_2$ (Figura 1/ Capítulo 1) pode assim se refletir em uma perda (parcial) da capacidade de reparo dos danos provocados no DNA. O aumento da sensibilidade ao Sn^{2+} de vários mutantes envolvidos em reparo (Viau *et al*., 2006) está na mesma ordem de magnitude que o observado para o *rnr4Δ*, bem como a falta de mutagênese induzida por UVC em *rnr4Δ* prejudicou a função de processos de reparo translesão sujeito a erro (Strauss *et al*., 2007).

Assim o mutante *rnr4Δ* apresentou uma maior sensibilidade que o WT para o $SnCl_2$ independente de sua fase celular (LOG/ STAT). Isto foi confirmado quando comparada a sensibilidade ao $SnCl_2$ do selvagem WT e do mutante *rnr4Δ*, onde o mutante *rnr4Δ* foi 3-4 vezes mais sensível que o isogênico WT (dados não apresentados). Com isso, a sensibilidade do mutante *rnr4Δ* ao Sn^{2+} é superior ao WT pois deve-se à sua menor geração de *pools* de dNTP o que dificulta o reparo de lesões no DNA induzido pelo $SnCl_2$.

6.2. Efeitos da ploidia no processo autofágico e resistência a mutagênicos em *Monilophthora perniciosa*

Não houve correlação entre a formação do tubo germinativo dos basidiósporos de *M. perniciosa* e sua viabilidade celular após 7 dias quando expostos aos mutagênicos (Figura 2 e 3/ Capítulo II), apesar da formação de pseudo-colônias estar diretamente ligada à germinação dos basidiósporos.

A aquisição de resistência das hifas dicarióticas de *M. perniciosa* dependente da fonte de carbono (glicose ou glicerol), que, no entanto, não é tão acentuada como nas hifas monocarióticas (Figura 3/ Capítulo II); essa aquisição de resistência aos mesmos mutagênicos quando células dicarióticas são pré-crescidas em glicerol e transferidas para glicose ou para glicerol também foi observada por Santos *et al*. (2008). Isso pode ser explicado por uma resposta ao

estresse oxidativo para a fonte de carbono não-fermentável, glicerol, e pela expressão de genes de defesa anti-ERO (Mp*CTT1* e Mp*CTA1*), que revela que a regulação depende da fonte de carbono, semelhante a repressão catabólica pela fonte de carbono, ou pela indução acentuada de transportadores ABC (Santos *et al*., 2008).

A utilização de material intracelular de hifas velhas que foi degradado também pode ser utilizado pelas novas hifas para o seu crescimento (Maheshwari, 2005). A expressão Mp*ATG8*, provável gene envolvido no processo autofágico (Nakatogawa *et al*. 2007), indica que a fonte de carbono exerce influência significativa sobre sensibilidade/ resistência celular para os diferentes mutagênicos. O Mp*ATG8* foi reprimido na presença de glicerol, durante a exposição ao H_2O_2 (Figura 4A/ Capítulo II) e 4NQO (Figura 4B/ Capítulo II), e induzida na presença de glicose (Figuras 4A e B/ Capítulo II) na fase dicariótica. Aparentemente a ausência de glicerol é um sinal da falta eminente de nutrientes. Isto poderia acionar a expressão de genes envolvidos no controle do processo autofágico, especialmente após a lesão (Figura 3/ Capítulo II), onde a taxa de reciclagem é necessária para remoção de possíveis proteínas danificadas.

Wei *et al*. (2004) afirma que o glicerol desempenha um papel importante na transferência de nutrientes das plantas infectadas para o patógeno. O glicerol presente no apoplasto celular pode ser prontamente absorvido pelo patogêno. Diferentemente da glicose, que requer elaborados sistemas de transporte de membrana, o glicerol pode penetrar na membrana do fungo por difusão simples (Gancedo *et al*., 1968). Sabe-se que os tecidos da planta, da zona periférica de infecção têm reduzida quantidade de glicerol (Wei *et al.,* 2004). Tomadas em conjunto, todas as evidências sugerem que o glicerol e, possivelmente os lipídios, poderiam desempenhar um papel significativo da resposta do fungo ao estresse oxidativo promovido pela planta, como pôde ser observada *in vitro* na resposta aos mutagênicos utilizados.

O glicerol pode ser utilizado como única fonte de carbono e energia por bactérias e fungos e tem sido caracterizado como significativo na existência metabólica em planta em uma concentração de 1-2 µmol/g de peso fresco (Gerber *et al*., 1988), e, assim, é possível que o glicerol seja utilizado pelo *M. perniciosa* como fonte de carbono para infectar o *T. cacao*. Os níveis de glicerol usados para a germinação de basidiósporos de *M. perniciosa* e para manter a

fase biotrófica *in vitro* são comparáveis aos observados por Scarpari *et al*. (2005), que mostrou elevados níveis de glicerol durante a vassoura verde no *T. cacao*, em comparação com tecidos sadios. Nesta fase da infecção, as hifas monocarióticas usam o glicerol como fonte de energia, e, por conseguinte, o processo autofágico não é necessário (Figura 4A e B, meio com glicerol/ Capítulo II), embora possa também gerar ERO, o glicerol também induz defesas a EROs (Ames *et al*., 1993), que conduz ao reforço da resistência a ERO (Figura 3A e B/ Capítulo II). Altos níveis de glicerol, na planta, por isso, podem manter a fase biotrófica durante o desenvolvimento da doença e contribuir para acentuar a resistência das células ao estresse.

Depois que esses níveis mais elevados de glicerol encontrados nos tecidos infectados são completamente esgotados nos tecidos da vassoura seca, a fase necrotrófica de *M. perniciosa* prevalece (Calle *et al*., 1982) e o processo de autofagia também podem ser necessário para obtenção de nutrientes (Figura 4A e B, meio com glicose/ Capítulo II). Assim a ausência de glicerol pode ser um sinal futuro da escassez de nutrientes e que pode induzir a dicariotização do fungo na planta como um passo decisivo para completar o ciclo sexual. Durante a fase necrotrófica a planta não apresenta mecanismo de defesa, e então o fungo não possui a necessidade de se proteger contra EROs e, portanto, o fungo dicariótico mostra maior sensibilidade para mutagênicos (Figura 3 A a D/ Capítulo II), Filho *et al.* (2006) e Santos *et al*. (2008). Esse processo ocorre durante a vassoura seca onde as hifas são submetidas à baixa concentração de nutrientes. Neste cenário, a autofagia poderia ser um processo paralelo no fungo para obter nutrientes por digestão do material das hifas, a fim de dar continuidade no ciclo sexual (Figura 4/ Capítulo II).

6.3. Seleção de mutantes de *Monilophthora perniciosa* canavanina resistentes

O sucesso da mutagênese UVC induzida em *M. perniciosa* (Figura 1/ Capítulo 3) revelou que o gene Mp*CAN1*, provável responsável pela captação de arginina e L-canavanina, tem uma resposta fenotípica ao agente UVC semelhante ao demonstrado na levedura *S. cerevisiae* por Gocke e Manney (1979) (Figura 2/

Capítulo 3). Surpreendentemente, o mutante *Mpcan1*^{R} mostrou-se mais resistente ao H_2O_2 quando comparado com a linhagem selvagem (Figura 3/ Capítulo 3).

Recentemente foi demonstrado que a resistência a canavanina pode estar associada a um complexo protéico denominado NAC (nascent polypeptide associated complex). NAC é abundante e filogeneticamente conservado entre as espécies sendo responsável pelo nível natural de resistência de L-canavanina e também está envolvido no controle da qualidade protéica (Andersen *et al*., 2007). Provavelmente *Mpcan1R* não é um mutante NAC, uma vez que os mutantes de *M. perniciosa* apresentaram-se resistentes ao H_2O_2 (agente oxidante também de proteínas).

Yan *et al*. (2005) revelou a expressão de NAC através de estudos de proteômica de raiz de arroz quando estas são induzidas ao estresse salino. Assim, como *Mpcan1*^{R} provavelmente é uma permease de membrana, a alteração da permeabilidade da membrana pode ser um sinal intracelular para que genes envolvidos em resposta ao estresse (inclusive NAC) estejam induzidos no mutante *Mpcan1*^{R}.

A prova final do não funcionamento do gene Mp*CAN1* responsável pela resistência a canavanina ainda não foi feita, mas que pode ser fornecida pela complementação heteróloga com o gene *CAN1* da levedura *S. cerevisiae* nos mutantes *Mpcan1*^{R}.

Apesar de todas as complicações claramente associado à resistência a L-canavanina para um defeito no locus *CAN1*, este sistema de seleção de mutação a frente tem-se revelado útil para os estudos de mutação em *M. perniciosa.*

Este sistema de mutação pode agora ser estudado com mais detalhes, por exemplo, utilizando agentes mutagênicos mais potentes, como etilmetanosulfonato (EMS), que demonstrou ser um excelente mutagênico, pois provoca elevada taxa de mutações induzidas e baixa citotoxicidade (van Zeeland *et al*., 1983).

7. Conclusões

A partir da análise dos resultados obtidos neste estudo conclui-se que:

- A sensibilidade do mutante *rnr4* de levedura *S. cerevisiae* ao agente químico $SnCl_2$, é independente da fase de crescimento (LOG ou STAT). Isto significa que outros mecanismos estão envolvidos, mais provavelmente o não-funcionamento parcial do reparo de DNA causado pelo baixo fornecimento de *pools* de dNTPs.

- A ploidia do fungo *M. perniciosa* influencia a sensibilidade ou resistência a agentes mutagênicos, onde as defesas anti-ERO durante a fase monocariótica do fungo são mais ativas que durante a fase dicariótica.

- Durante a fase dicariótica, o fungo *M. perniciosa* utiliza nutrientes providos da ativação de genes envolvidos no processo autofágico (Mp*ATG8*).

- Testes para a avaliação da capacidade de inibição do tubo de germinação dos basidiósporos frente a agentes mutagênicos, mostraram a não correlação entre a formação do tubo germinativo e viabilidade celular do fungo *M. perniciosa*.

- A mutagenização de basidiósporos de *M. perniciosa* utilizando os mesmos conceitos genéticos do organismo modelo *S. cerevisiae* é factível, embora a resposta fisiológica obtida foi inesperada. Os mecanismos que envolvem esta resposta ainda não foram elucidados.

8. Perspectivas

Este trabalho abre novas possibilidades de aprofundamento dos dados aqui obtidos sobre sensibilidade e mutabilidade nos fungos *M. perniciosa* e *S. cerevisiae*, através das seguintes perspectivas:

- Buscar genes responsáveis pelo transporte do Sn^{2+} para o interior celular da levedura *S. cerevisiae*, testando linhagens defectivas no transporte de membrana, do tipo ABC (transportadores com ATP-*binding cassette*);

- Identificar a localização do Sn^{2+} no interior celular da levedura *S. cerevisiae*, com a utilização de microscopia eletrônica de transmissão;

- Observar o processo de mudança de fase do fungo *M. perniciosa*, através da definição de genes envolvidos na dicariotização, assim como a caracterização fenotípica e molecular deste processo;

- Dar continuidade ao trabalho de mutagenização dos basidiósporos de *M. perniciosa* para obtenção de novos mutantes de *CAN1*, repetindo-se os experimentos já realizados, além da posterior caracterização dos mutantes *can1*^{R}, bem como a mutação do gene Mp*URA3,* resultando num fenótipo de resistência para 5-ácido fluorótico (5-FOA) e auxotrofia para uracil.

9. Referências Bibliográficas

Abia - Associação Brasileira das Indústrias da Alimentação. Portaria n^{o}685, de 27 de agosto de 1998. Secretaria de Vigilância Sanitária. Regulamento Técnico: "Princípios Gerais para o Estabelecimento de Níveis Máximos de Contaminantes Químicos em Alimentos". Diário Oficial, Brasília, 24 set. 1998, Secção 1.

Aime and Phillips-Mora. 2005. The causal agents of witches' broom and frosty pod rot of cacao (chocolate, *Theobroma cacao*) form a new lineage of Marasmiaceae. Mycologia 97:1012-22.

Akaogi J, Barker T, KurodaY, Nacionales DC, YamasakiY, Stevens BR, Reeves WH, Satoh M. 2006. Role of non-protein amino acid L-canavanine in autoimmunity. Autoimmunity Reviews 5:429-435.

Alexopoulos, CJ. 1996. Introductory mycology. U.S.A., John Wiley & Sons, Inc.

Ames BN, Shigenaga MK, Hagen TM. 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences of the United States of America 90:7915-7922.

Anderbrhan T, Hammerstone JF, Romanczyk LJ, Furtek DB. 1995. Sensitivity of *Crinipellis perniciosa* to procyanidins from *Theobroma caco* L. Physiological and a Molecular Plant Pathology 46:339-348.

Andersen M, Sample CA, Hartmann-Petersen R. 2007. Characterization of the nascent polypeptide-associated complex in fission yeast. Molecular Biology Reports 34:275-281.

Apel K, Hirt H. 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55:373-399.

Ashby J, Tennant RW. 1991. Definitive relationships among chemical structure, carcinogenicity and mutagenicity for 301 chemicals tested by the U.S. NTP. Mutation Research 257:229-306.

Assis MLB, Dantas FJS, Caldeira-De-Araújo A, Bernardo-Filho M. 1998a. Stannous chloride and glucoheptonic acid: effect of a kit used in nuclear medicine. Cancer Letters 130:127-131.

Assis MLB, Caceres MR, de Mattos JCP, Caldeira-De-Araujo A, Bernardo-Filho M. 1998b. Cellular inactivation induced by a radiopharmaceutical kit: role of stannous chloride. Toxicology Letters 99:199-205.

Ayaki H, Lee MJ, Sumino K, Nishio H. 2005. Different cytoprotective effect of antioxidants and change in the iron regulatory system in rodent cells exposed to paraquat or formaldehyde. Toxicology 2081:73-79.

Basso TS, Pungartnik C, Brendel M. 2008. Low productivity of ribonucleotide reductase in *Saccharomyces cerevisiae* increases sensitivity to stannous chloride Genetics and Molecular Research 7:1-6.

Bastos CN, Andebrhan T. 1986. Urucum (*Bixa orellana*): nova espécie hospedeira da vassoura-de-bruxa (*Crinipellis perniciosa*) do cacaueiro. Fitopatologia Brasileira 11:963-965.

Bernardo-Filho M, Cunha MC, Valsa JO, Caldeira-de-Araújo A, Silva FCP, Fonseca AS. 1994. Evaluation of potential genotoxicity of stannous chloride: inactivation, filamentation and lysogenic induction of *Escherichia coli*. Food and Chemical Toxicology 32:477-479.

Blunden S, Wallace T. 2003. Tin in canned food: a review and understanding of occurrence and effect. Food and Chemical Toxicology 41:1651-1662.

Bonneh-Barkay D, Reaney SH, Langston WJ, Di Monte DA. 2005. Redox cycling of the herbicide paraquat in microglial cultures. Molecular brain research 1341:52-56.

Boveris A. 1998. Biochemistry of free radicals: from electrons to tissues. Medicina (Buenos Aires) 58:350-356.

Bruns, TD, Vilgalys R, Barns SM, Gonzalez D, Hibbett DS, Lane DJ, Simon L, Stickel S, Szaro TM, Weisburg WG, Sogin ML. 1993. Evolutionary relationships within the fungi: analysis of nuclear small subunit rRNA sequences. Molecular Phylogenetics and Evolution 1:231-241.

Bruns TD, White TJ, Taylor JW. 1991. Fungal molecular systematics. Annual Review of Ecology and Systematics 22:525-564.

Burba JV. 1983. Inhibition of hepatic azo-reductase and aromatic hydroxylase by radiopharmaceuticals containing tin. Toxicology Letters 18:269-72.

Cabral REC, Leitão AC, Lage C, Caldeira-de-Araújo A, Bernardo-Filho M, Dantas FFS, Cabral-Neto J. 1998. Mutational potentiality of stannous chloride: an important reducing agent in the 99mTcradiopharmaceuticals. Mutation Research 408:129-135.

Calle H, Cook AA, Fernando SY. 1982. Histology of witches´ broom caused in cacao by *Crinipellis perniciosa*. Phytopathology 72:1479-1481.

Carlile MJ, Watkinson SC. 1996. The fungi. Academic Press Ltd. 3ª Edition. Londres.

Ceita GO, Macêdo JNA, Santos TB, Alemanno L, Gesteira AS, Micheli F, Mariano AC, Gramacho KP, Silva DC, Meinhardt L, Mazzafera P, Pereira GAG, Cascardo JCM. 2007. Involvement of calcium oxalate degradation during programmed Q1 cell death in *Theobroma cacao* tissues triggered by the hemibiotrophic fungus *Moniliophthora perniciosa*. Plant Science (Limerick) 173:106-117.

Chmielnicka J, Szymanska JA, Sniec. 1981. Distribution of tin in the rat and disturbances in the metabolism of zinc and copper due to repeated exposure to $SnCl_2$. Archives of Toxicology 47:263-268.

Costa V, Ferreira PM. 2001. Oxidative stress and signal transduction in *Saccharomyces cerevisiae*: insights into ageing, apoptosis and diseases. Molecular Aspects of Medicine 22:217-246.

Cotomacci C. 2004. Análise genômica do sistema *mating type* de *Crinipellis perniciosa*, fungo causador da vassoura-de-bruxa em *Theobroma cacao*. Campinas - SP: Universidade Estadual de Campinas. 75p.

Dantas FJ, Moraes MO, de Mattos JC, Bezerra RJ, Carvalho EF, Filho MB, Caldeira de Araujo A. 1999. Stannous chloride mediates single strand breaks in plasmid DNA through reactive oxygen species formation. Toxicology Letters 110:129-136.

Dantas FJS, Moraes MO, Carvalho EF, Valsa JO, Bernardo-Filho M, Caldeira-de-Araújo A. 1996. Lethality induced by stannous chloride on *Escherichia coli* AB1157: participation of reactive oxygen species. Food and Chemical Toxicol 34:959-962.

de Arruda MCC, Ferreira MASV, Miller RNG, Resende MLV, Felipe MSS. 2003a. Nuclear and mitochondrial rDNA variability in *Crinipellis perniciosa* from different geographic origins and hosts. Mycological Research 107:25-37.

de Arruda MCC, Miller RNG, Ferreira MASV, Felipe MSS. 2003b. Comparison of *crinipellis perniciosa* isolates from Brazil by ERIC repetitive element sequence-based PCR genomic fingerprinting. Plant Pathology 52:236-244.

de Groot AP, Feron VJ, Til HP. 1973. Short-term toxicity studies on some salts and oxides of tin in rats. Food and Cosmetics Toxicology 11:19-30.

de Mattos JC, Dantas FJ, Bezerra RJ, Bernardo-Filho M, Cabral-Neto JB, Lage C, Leitão AC, Caldeira-de-Araujo A. 2000. Damage induced by stannous chloride in plasmid DNA. Toxicology Letters 116:159-163.

de Vries S, Marres CAM. 1988. The mitochondrial respiratory chain of yeast structure and biosynthesis and the role in cellular metabolism. Biochimica et Biophysica Acta 895:205-239.

Decottignies A, Grant AM, Nichols JW, de Wet H, McIntosh DB, Goffeau A. 1998. ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. Journal of biological chemistry 273:12612-12622.

Delgado JC, Cook AA. 1976. Nuclear condition of the basidia, basidiosporos, and mycelium of *Marasmius perniciosus*. Canadian Journal of Botany 54:66-72.

Dias LAS. 2001. Melhoramento Genético do Cacaueiro. Viçosa - MG: FUNAP, UFG. 578p.

Dickinson RJ, Schweizer M. 1999. The metabolism and molecular physiology of *Saccharomyces cerevisiae*. T.J. International Ltd. Padstow, UK.

Dizotti CE, Coutinho SD. 2007. Isolation of Malassezia pachydermatis and M. sympodialis from the external ear canal of cats with and without otitis externa. Acta veterinaria Hungarica 471-477.

Ekmekci Y, Terzioglu S. 2005. Effects of oxidative stress induced by paraquat on wild and cultivated wheats. Pesticide Biochemistry and Physiology 1-13.

Elledge SJ, Davis RW. 1987. Identification and isolation of the gene encoding the small subunit of ribonucleotide reductase from *Saccharomyces cerevisiae*: DNA damage-inducible gene required for mitotic viability. Molecular and Cellular Biology 7:2783-2793.

Elledge SJ, Zhou Z, Allen JB, Navas TA. 1993. DNA damage and cell cycle regulation of ribonucleotide reductase. Bioessays 15:333-339.

Evans HC, Bastos CN. 1979. Uma avaliação do ciclo de vida da vassoura de bruxa (*Crinipellis perniciosa*) do cacaueiro. Fitopatologia Brasileira 41:515-523.

Evans HC. 1980. Pleomorphism in *Crinipellis perniciosa*, causal agent of witches' broom disease of cocoa. Transactions of the British Mycological Society 74:515-523.

Fang GC, Hanau RM, Vaillancourt LJ. 2002. The *SOD2* gene, encoding a manganese-type superoxide dismutase, is up-regulated during conidiogenesis in the plant–pathogenic fungus *Colletotrichum graminicola*. Fungal Genetics and Biology 36:155-165.

Fernández-Benéeitez MA, Ortiz-Santaliestra MO, Lizana M, Diéguez- Uribeondo J. 2008. *Saprolegnia diclina*: another species responsible for the emergent disease 'Saprolegnia infections' inamphibians. FEMS Microbiology Letters 279:23-29.

Filho DF, Pungartnik, Cascardo JCM, Brendel M. 2006. Broken hyphae of the basidiomycete *Crinipellis perniciosa* allow quantitative assay of toxicity. Current Microbiology 52:407-412.

Forman HJ, Thomas MJ. 1986. Oxidant production and bactericidal activity of phagocytes. Annual Review of Physiology 48:669-680.

Frias GA, Purdy LH, Schmidt RA. 1991. Infection biology of *Crinipellis perniciosa* on vegetative flushes of cacao. Plant Disease 7:552-556.

Fridovich I. 1998. Oxygen toxicity: a radical explanation. The Journal of Experimental Biology 201:1203-1209.

Friedberg EC, Walker GC, Siede W. 1995. DNA Repair and Mutagenesis. Washington, D.C., American Society for Microbiology press.

Fuge EK, Werner-Washburne M. 1997. Stationary phase in the yeast *Saccharomyces cerevisiae*. In: HOHMANN, S and MAYER, W. H. (Eds) Yeast Stress Response, Heidelberg: Springer-Verlag, 53-74.

Gancedo C, Gancedo JM, Sols A. 1968. Glycerol metabolism in yeasts. Pathways of utilization and production. European Journal Biochemistry 5:165-172.

Gancedo JM. 1998. Yeast carbon catabolite repression. Microbiology and Molecular Biology Reviews 62:334-361.

Garfinkel DJ. 2005. Genome evolution mediated by Ty elements in *Saccharomyces*. Cytogenetic and Genome Research 110:63-69.

Gerber DW, Byerrum RU, Gee RW, Tobert NE. 1988. Glycerol concentrations in crop plants. Plant Science 56:31-38.

Gesteira AS, Micheli F, Ferreira CF, Cascardo JC. 2003. Isolation and purification of functional total RNA from different organs of cacao tree during its interaction with the pathogen *Crinipellis perniciosa*. Biotechniques 35:494-6, 498-500.

Gocke E, Manney TR. 1979. Expression of radiation-induced mutations at the arginine permease (*CAN1*) locus in *Saccharomyces cerevisiae*. Genetics 91:53-66.

Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG. 1996. Life with 6.000 genes. Science 274:546, 563-7.

Goffeau A. 2000. Four years of post-genomic life with 6.000 yeast genes. FEBS Letters 4801:37-41.

Greger JL, Baier M. 1981. Tin and iron content of canned and bottled food. Journal and Food Science 46:1751-1765.

Griffith GW, Hedger JN. 1994. The breeding biology of biotypes of the witches' broom pathogen of cocoa, *Crinipellis perniciosa*. Heredity 72:278-289.

Griffith GW. 2004. Witches' broom and frosty pods. Biologist 51:71-75.

Griffth GW, Bravo-Velasquez E, Wilson FJ, Lewis DM, Hedger JN. 1994. Autecology and evolution of the wicthes broom pathogen (*Crinipellis perniciosa*) of cocoa. In. Blakeman JP, Williamson B. (Ed.). Ecology of Plant Pathogens. Oxon: CAB Internacional. 245-267.

Hamasaki T, Sato T, Nagase H, Kito H. 1993. The mutagenicity of organotin compounds as environmental pollutants. Mutation Research 300:265-271.

Ikem A, Egiebor NO. 2005. Assessment of trace elements in canned fishes (mackerel, tuna, salmon, sardines and herrings) marketed in Georgia and Alabama (United States of America). Journal of Food Composition and Analysis 18:771-787.

Johnson MA, Greger JL. 1982. Effects of dietary tin on tin and calcium metabolism of adult males. The American Jurnal of Clinical Nutrition 35:655-660.

Johnston M, Kim JH. 2005. Glucose as a hormone: receptor-mediated glucose sensing in the yeast *Saccharomyces cerevisiae*. Biochemical Society Transactions 33:247-52.

Johnston M. 1999. Feasting, fasting and fermenting. Glucose sensing in yeast and other cells. Trends in Genetics: TIG 15:29-33.

Kaniak A, Xue Z, Macool D, Kim JH, Johnston M. 2004. Regulatory network connecting two glucose signal transduction pathways in *Saccharomyces cerevisiae*. Eukaryotic Cell 3:221-31.

Kappus H, Sies H. 1981. Toxic drug effects associated with oxygen metabolism: redox cycling and peroxidation. Experientia 37:1233-1241.

Kaszycki P, Czechowska K, Petryszak P, Międzobrodzki J, Pawlik B, Kołoczek H. 2006. Methylotrophic extremophilic yeast *Trichosporon* sp.: a soil-derived isolate with potential applications in environmental biotechnology. Acta Biochimica Polonica 53:463-473.

Kurjan, J. 1992. Pheromone response in yeast. Annual Review of Genetic 61:1097-1129.

Kurjan, J. 1993. The pheromone response pathway in *Saccharomyces cerevisiae*. Annual Review of Genetic 27:147-179.

Landis GN, TOWER J. 2005. Superoxide dismutase evolution and life span regulation. Mechanisms of Ageing and Development 126:365-379.

Lehmann AR. 2000. Replication of UV – damage DNA: new insights into links between DNA polymerases, mutagenesis and human diseases. Gene 253:1-12.

Liti G, Louis EJ. 2005. Yeast evolution and comparative genomics. Annual Review of Microbiology 59:135-153.

Luzzi SD, Marletta MA. 2005. L-Arginine analogs as alternate substrates for nitric oxide synthase. Bioorganic & Medicinal Chemistry Letters 15:3934-3941

Maheshwari R. 2005. Nuclear behavior in fungal hyphae. FEMS Microbiology Letters 249:7-14.

Maris AF, Kern AL, Picada JN, Boccardi F, Brendel M, Henriques JAP. 2000. Glutathione, but not transcription factor Yap1, is required for carbon source-dependent resistance to oxidative stress in *Saccharomyces cerevisiae*. Current Genetics 37:175-182.

Maron DM, Ames BN. 1983. Revised methods for the Salmonella mutagenicity test. Mutation Research 113:173-215.

McLean JRN, Blakey DH, Douglas GR, Kaplan JG. 1983a. The effect of tin chloride on the structure and function of DNA in human white blood cells. Chemico-Biological Interactions 119:195-201.

McLean JRN, Birnboim HC, Pontefact R, Kaplan JG. 1983b. The effect of stannous and stannic (tin) chloride on DNA in Chinese hamster ovary cells. Mutation Research 119:195-201.

Meinhardt LW, Bellato Cde M, Rincones J, Azevedo RA, Cascardo JC, Pereira GA. 2006. *In vitro* production of biotrophic-like cultures of *Crinipellis perniciosa*, the causal agent of witches broom disease of *Theobroma cacao*. Current Microbiology 52:191-196.

Melo SCO, Pungartnik C, Cascardo JCM, Brendel M. 2006. Rapid and efficient protocol for DNA extraction and molecular identification of the basidiomycete *Crinipellis perniciosa*. Genetics and Molecular Research 5:851-855.

Micheli F. 2003. Bases genéticas da resistência do cacaueiro à doença de vassoura da bruxa (*Crinipellis perniciosa*). In: O Cirad no Brasil. Relatório de Atividades 2001-2002. Brasilia: CIRAD, 22-23.

Mucheroni ML, Matias VR. 1996. Análise de rugosidade de fungos através de dimensão fractal. Anais do IX SIBGRAPI. Caxambu-MG**.** 351-352.

Nakatogawa H, Ichimura Y, Ohsumi Y. 2007. Atg8, a ubiquitin-like protein required for autophagosome formation, mediates membrane tethering and hemifusion. Cell 130:165-78.

Nelson DL, Cox MM. 2002. Lehninger Princípios de Bioquímica. Ed Sarvier, 3ª ed. São Paulo.

Nickoloff JA, Hoekstra M. 1998. DNA Damage and Repair: DNA Repair in Prokaryotes and Lower Eukaryotes. Vol.1. Human Press. Totowa, New Jersey.

Oga Z. 2003. Fundamentos de toxicologia. Ed. Atheneu. 2ª.ed. São Paulo. 39-44.

Olivarius FF, Balslev E, Menné T. 1993. Skin reactivity to tin chloride and metallic tin. Contact Dermatitis 29:110-111.

Olivier P, Marzin D. 1987. Study of the genotoxic potential of 48 inorganic derivatives with the SOS chromotest. Mutation Research 189:263-269.

Orchard J, Collin HA, Hardwick K, Isaac S. 1994. Changes in morphology and measurement of cytokin levels during the development of witches' brooms on cocoa. Plant Pathology 43:65-72.

Pereira JL, Almeida LCC, Santos SM. 1996. Witches' broom disease of cocoa in Bahia: attempts at eradication and containment. Crop Protection 15:743-752.

Perlstein DL, Ge J, Ortigosa AD, Robblee JH, Zhang Z, Huang M, Stubbe J. 2005. The active form of the *Saccharomyces cerevisiae* ribonucleotide reductase small subunit is a heterodimer *in vitro* and *in vivo*. Biochemistry 44:15366-15377.

Peter PS, Wartena M, Kampinga HH, Konings AWT. 1992. Role of lipid peroxidation and DNA damage in paraquat with ionizing radiation. Biochemical Phamacology 43:705-715.

Pungartnik C, Viau C, Picada J, Caldeira-de-Araújo A, Henriques JA, Brendel M. 2005. Genotoxicity of stannous chloride in yeast and bacteria. Mutation Research 583:146-157.

Purdy L, Hand Schmidt RA. 1996. Status of cacao witches' broom: biology, epidemiology and management. Annual Review of Phytopathology 34:573-594.

Queiroz VT, Guimarães CT, Anhert D, Schuster I, Daher RT, Pereira MG, Miranda VRM, Loguercio LL, Barros EG, Moreira MA. 2003. Identification of a major QTL in cocoa (*Theobroma cacao* L.) associated with resistance to witches´ broom disease. Plant Breeding 122:268-272.

Resende MLV, Nojosa GBA; Silva LHCP, Niella GR, Carvalho GA, Santiago DVR, Bezerra JL. 2000. *Crinipellis perniciosa* proveniente de um novo hospedeiro, *Heteropterys acutifólia*, é patogênico ao cacaueiro. Fitopatologia Brasileira 25:88-91.

Rincones J, Meinhardt LW, Vidal BC, Pereira GA. 2003. Electrophoretic karyotype analysis of *Crinipellis perniciosa*, the causal agent of witches broom disease of *Theobroma cacao*. Mycological Research 107:452-458.

Rocha HM, Wheller BEJ. 1985. Factors influencing the prodution of basidiocarps and deposition and germination of basidiospores of *Crinipellis perniciosa*, the casual fungus of witches´ broom on cocoa (*Theobroma cacao*). Plant Pathology 34:319-328.

Rodriguez CE, Sobol Z, Schiestl RH. 2007. 9,10-Phenanthrenequinone induces DNA deletions and forward mutations via oxidative mechanisms in the yeast *Saccharomyces cerevisiae*. Toxicology in Vitro. In Press

Rolland F, Winderickx J, Thevelein JM. 2002. Glucose-sensing and -signalling mechanisms in yeast. FEMS Yeast Research 2:183-201.

Rosenthal GA. 1977. The biological effects and mode of action of L-canavanine, a structural analogue of L-arginine. The Quarterly Review of Biology 52:155-78.

Rosenthal GA. 2001. L-canavanine: a higher plant insecticidal allelochemical. Amino Acids 21:319-30.

Rüdel, H. 2003. Case study: bioavailability of tin and tin compounds. Ecotoxicology and Environmental Safety 56:180-189.

Sakai S, Minoda K, Saito G, Fukuoca, F. 1955. Gann; the Japanese Journal of Cancer Research 46:605.

Santos RX, Melo SCO, Cascardo JCM, Brendel M, Pungartnik C. 2008. Carbon source-dependent variation of acquired mutagen resistance of *Moniliophthora perniciosa*: similarities in natural and artificial systems. Fungal Genetics and Biology. Accepted.

Scarpari LM, Meinhardt LW, Mazzafera P, Pomella AW, Schiavianato MA, CascardoJC, Pereira GA. 2005. Biochemical changes during the development of witches' broom: the most important disease of cocoa in Brazil caused by *Crinipellis perniciosa*. Journal of Experimental Botany 56:865-877.

Schroeder HA, Balassa JJ, Tripton IH. 1964. Abnormal trace metals in man: tin. Journal of Chronic Diseases 17:483-502.

Silva J, Erdtmann B, Henriques JAP. 2003. Genética Toxicológica. Ed. Alcance. Porto Alegre.

Silva SDV, Luz EDMN, Almeida OC, Gramacho KP, Bezerra JL. 2002. Redescrição da sintomatologia causada por *Crinipellis perniciosa* em cacaueiro. Agrotrópica 14:1-28.

Silva SDVM. 1997**.** Histologia e seleção de variáveis para avaliar resistência de cacaueiro a *Crinipellis perniciosa***.** Viçosa-MG: Universidade Federal de Viçosa. 93p.

Snustad DP, Simmons MJ. 2001. Fundamentos de Genética. Ed. Guanabara Koogan. 2ª ed. Rio de Janeiro.

Strauss M, Grey M, Henriques JAP, Brendel M. 2007. *RNR4* mutant alleles *pso3-1* and *rnr4Δ* block induced mutation in *Saccharomyces cerevisiae*. Current Genetics 51:221-31.

Stubbe J, Van Der Donk WA. 1998. Protein radicals in enzyme catalysis. Chemical Reviews 98:705-762.

Sugimura T, OkabeM, Nagão M. 1966. The metabolism of 4-nitroquinoline-1-oxide, a carcinogen. 3. An enzyme catalyzing the conversion of 4-nitroquinoline-1-oxide to 4-hydroxyaminoquinoline-1-oxide in rat liver and hepatomas. Cancer Research 26:1717-1721.

Tada e Tada, 1976. Main binding sites of the carcinogen, 4 nitroquinolina 1-oxide, in a subecellular microssomal system. Chemico-Biological Interactions 29:257-266.

Thorold .A. 1975. Diseases of cacao. Claredon Press, Oxford, U.K.

van Zeeland AA, Mohn GR, Aaron CS, Glickman BW, Brendel M, de Serres FJ, Hung CY, Brockman HE. 1983. Molecular dosimetry of the chemical mutagen ethyl methanesulfonate. Quantitative comparison of the mutagenic potency in *Neurospora crassa* and *Saccharomyces cerevisiae*. Mutation Research 119:45-54.

Viau C, Pungartnik C, Schmitt MC, Basso TS, Henriques JAP, Brendel M. 2006. Sensitivity to Sn^{2+} of the yeast *Saccharomyces cerevisiae* depends on general energy metabolism, metal transport, anti-oxidative defenses, and DNA repair. BioMetals 19:705-714.

von Mayenburg J, Rakoski J, Szliska C. 1991. Patch testing with amalgam in various concentrations. Contact Dermtitis 24:266-269.

Vultaggio A, Lombardelli L, Giudizi MG, Biagiotti R, Mazzinghi B, Scaletti C, Mazzetti M, Livi C, Leoncini F, Romagnani S, Maggi E, Piccinni MP. 2007. T cells specific for *Candida albicans* antigens and producing type 2 cytokines in lesional mucosa of untreated HIV-infected patients with pseudomembranous oropharyngeal candidiasis. Microbes and Infection [Epub ahead of print]

Wei Y, Shen W, Dauk M, Wang F, Selvaraj G, Zou J. 2004. Targeted gene disruption of glycerol-3-phosphate gehydrogenase in *Colletotrichum gloeosporioides* reveals evidence that glycerol is a significant transferred nutrient from host plant to fungal pathogen. The Journal of Biological Chemistry 279:429-435.

Wills C. 1990. Regulation of sugar and ethanol metabolism in *Saccharomyces cerevisiae*. Critical Reviews in Biochemistry and Molecular Biology 25:245-280.

Yan S, Tang Z, Su W, Sun W. 2005. Proteomics analysis of salta stress-responsive proteins in rice root. Proteomics 5:235-44.

Zimmermann FK, von Borstel RC, Von Halle ES, Parry JM, Siebert D, Zetterberg G, Barale R, Loprieno N. 1984. Testing of chemicals for genetic activity with *Saccharomyces cerevisiae*: a report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation Research 133:199-244.

ANEXO

BioMetals (2006) 19:705–714
DOI 10.1007/s10534-006-9007-1

Sensitivity to Sn^{2+} of the yeast *Saccharomyces cerevisiae* depends on general energy metabolism, metal transport, anti-oxidative defences, and DNA repair

C. Viau[1], C. Pungartnik[2], M.C. Schmitt[1], T.S. Basso[2], J.A.P. Henriques[1] & M. Brendel[2,*]
[1]*Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, UFRGS, Porto Alegre, RS, Brasil;* [2]*Departamento de Biology, Universidade Estadual de Santa Cruz (UESC), Rodovia Ilhéus-Itabuna Km 16, CEP-45662-000, Ilhéus, Bahia, Brasil; *Author for correspondence (E-mail: martinbrendel@yahoo.com.br)*

Received 10 October 2005; accepted 18 March 2006

Key words: cytochrome oxidase, DNA repair, genotoxicity, glucose repression, membrane transport, oxidative stress, stannous chloride, yeast

Abstract

Resistance to stannous chloride ($SnCl_2$) of the yeast *Saccharomyces cerevisiae* is a product of several metabolic pathways of this unicellular eukaryote. Sensitivity testing of different null mutants of yeast to $SnCl_2$ revealed that DNA repair contributes to resistance, mainly via recombinational (Rad52p) and error-prone (Rev3p) steps. Independently, the membrane transporter Atr1p/Snq1p (facilitated transport) contributed significantly to Sn^{2+}-resistance whereas absence of ABC export permease Snq2p did not enhance sensitivity. Sensitivity of the superoxide dismutase mutants *sod1* and *sod2* revealed the importance of these anti-oxidative defence enzymes against Sn^{2+}-imposed DNA damage while a catalase-deficient mutant (*ctt1*) showed wild type (WT) resistance. Lack of transcription factor Yap1, responsible for the oxidative stress response in yeast, led to 3-fold increase in Sn^{2+}-sensitivity. While loss of mitochondrial DNA did not change the Sn^{2+}-resistance phenotype in any yeast strain, cells with defect cytochrome *c* oxidase (CcO mutants) showed gradually enhanced sensitivities to Sn^{2+} and different spontaneous mutation rates. Highest sensitivity to Sn^{2+} was observed when yeast was in exponential growth phase under glucose repression. During diauxic shift (release from glucose repression) Sn^{2+}-resistance increased several hundred-fold and fully respiring and resting cells were sensitive only at more than 1000-fold exposure dose, i.e. they survived better at 25 mM than exponentially growing cells at 25 μM Sn^{2+}. This phenomenon was observed not only in WT but also in already Sn^{2+}-sensitive *rad52* as well as in *sod1*, *sod2* and CcO mutant strains. The impact of metabolic steps in contribution to Sn^{2+}-resistance had the following ranking: Resting WT cells > membrane transporter Snq1p > superoxide dismutases > transcription factor Yap1p ≥ DNA repair ≫ exponentially growing WT cells.

Introduction

Trace amounts of different metals play a crucial role in cellular metabolism as they constitute ligands of diverse enzymes (Eide & Guerinot 1997). At higher concentrations metal ions, especially some of heavy metals, interfere negatively with cellular metabolism as they may inactivate proteins and damage DNA (McMurray & Tainer 2003). Evolution thus favoured survival of organisms which had developed mechanisms that guaranteed optimal intracellular metal concentration by balancing metal uptake from the environment and metal excretion/neutralization (metal homeostasis (Tomsett & Thurmann 1988)). Modern food preservation relies on sterilization and

packaging of food, and tin plays an important role in this process as it is used for the inner lining of metal containers and for conservation of soft drinks (McLean *et al*. 1983a). Tin thus comes into contact with the packaged food and may form stannous salts. Increased consumption of canned foods, therefore, is held responsible for tin accumulation in humans of wealthy countries (Schroeder *et al*. 1964).

Stannous chloride ($SnCl_2$) is a weak mutagen as defined by its genotoxicity in unicellular prokaryotes (Bernardo-Filho *et al*. 1994; Dantas *et al*. 1996) and eukaryotes (Pungartnik *et al*. 2005) as well as its DNA interactions in mammalian cells (McLean & Kaplan 1979; McLean *et al*. 1983b). Mutagenicity and mitotic gene conversion induced by Sn^{2+} in *Saccharomyces cerevisiae* points to the involvement of error-prone repair mechanisms in the removal of DNA lesions and the involvement in repair of the recombinational Rad52-controlled pathway was also shown (Pungartnik *et al*. 2005).

Metal uptake and metal homeostasis in yeast are controlled by a complex system of metabolic steps, most prominently by membrane transporters (Eide & Guerinot 1997; Van Ho *et al*. 2002) and by intracellular neutralization with thiol-oligopeptides and metallothioneins (Heuchel *et al*. 1994). Thus it is likely that changes in Sn^{2+} uptake or excretion via membrane transporters might also influence sensitivity of yeast cells to this metal. Once in the cell, the genotoxic potential of Sn^{2+} might also be significantly modulated by other, non-DNA repair or membrane transport-related physiological parameters, i.e., the quality and quantity of enzymatic and non-enzymatic scavengers of metal-induced reactive oxygen species (ROS). $SnCl_2$ is known to produce ROS (Dantas *et al*. 1999) most probably via Fenton-like reactions (McLean *et al*. 1983b) and thus the genetic endowment of yeast with anti-oxidative defence systems, e.g., superoxide dismutases, catalase, glutathione, and their oxidative stress-induced expression might contribute to Sn^{2+}-resistance.

Anaerobically growing microorganisms, especially obligate anaerobes, are known to have a higher metal sensitivity than aerobically living microbial species. The facultative anaerobe yeast *S. cerevisiae* can grow both in presence or absence of respiratory metabolism, and thus might be a good model organism to test the influence of general energy metabolism on sensitivity to Sn^{2+}. Mitochondrial activity plays a crucial role in aerobic energy metabolism of eukaryotes and it is thus likely that defects in the respiratory chain located within the inner mitochondrial membrane might directly or indirectly contribute to the generation of ROS (Barros *et al*. 2003) which might be altered by the presence of Sn^{2+}.

All above-mentioned metabolic steps are controlled by proteins encoded in the yeast cell's genome and thus can be influenced by genetic manipulation. Therefore, this unicellular fungus offers itself as an ideal eukaryotic model for the observation of Sn^{2+}-induced effects on its DNA, allowing to determine the relative contribution to Sn^{2+}-resistance of individual protective metabolic pathways.

Materials and methods

Yeast strains and growth conditions

The relevant genotypes of the yeast strains used in this work are given in Table 1. Media, solutions and buffers were prepared according to Burke *et al*. (2000). Complete medium (YPD) was used for routine growth of yeast cells and minimal medium (MM) was supplemented with the appropriate amino acids (synthetic complete medium, SC). To ascertain yeast respiratory competence and for elimination of spontaneously accumulated *petites* all strains were pre-grown on YPglycerol media (glucose replaced by 2% glycerol) before being grown in YPD.

Yeast exposure to $SnCl_2$ and survival

Stationary (STAT) cells were grown in YPD at 30 °C for 72 h. Different times of growth of STAT cells in fresh medium yielded cells in exponentially phase of growth (LOG). LOG cells were microscopically checked for bud appearance and the bud index (% budded cells) was established. Sensitivity of twice saline-washed yeast suspensions to $SnCl_2$ was routinely determined in liquid saline (0.9% NaCl, pH 5.0). Exposure concentration was 25 mM for STAT cells and 25 μM for LOG cells. Exposure time was for 60 min at 30 °C. Thereafter, $SnCl_2$-mediated cell aggregates were de-clumped in phosphate buffer (PB, pH 7.4, 0.067 M) followed by vigorous vortexing before

Table 1. Strains used in this study and their relevant genotypes.

Yeast strains	Genotype	Reference
XS2316 (WT)	*MAT***a** + *leu1 − 1trp5 − 48 + + + his1 − 208* *MATα ade6 leu1-12 + cyh2 met13 lys5-1 his1-208*	Machida & Nakai (1980)
XV185-14c (WT)	*MATα ade2-2 his1-798 lys1-1 trp5-48 hom3-10 arg4-17*	von Borstel *et al.* (1971)
BY10000 (WT)	*MATα his3*Δ1 *lys2*Δ0 *leu2*Δ0 *ura3*Δ0	EUROSCARF
4 BY *rad* mutants	Same genotype as BY10000 but *rad52*Δ, *rad2*Δ, *rad4*Δ, *rad6*Δ	See above
YPH98 (WT)	*MAT***a** *ura3-52 lys2-801 ade2-101 leu2-*Δ*1 trp1-*Δ*1*	Wehner *et al.* (1983)
4NQO sensitive	Same genotype as YPH98 but *snq1*Δ, *snq2*Δ, *snq3*Δ/*yap1*Δ	See above
q1	Same genotype as YPH98 but rho^0	M. Grey, Frankfurt/Main
q2	Same genotype as q1 but *gsh1*Δ	See above
q3	Same genotype as q1 but *gsh1*Δ*lwg1*Δ	See above
q4	Same genotype as q1 but *lwg1*Δ	See above
EG103 (WT)	*MATα* it leu2-3, 112 his3Δ*1 trp1-289 ura3-52 GAL*$^+$	E.B. Gralla, Los Angeles
EG118 (*sod1*Δ)	*sod1::URA3* all others markers as EG103	See above
EG110 (*sod2*Δ)	*sod2::TRP1* all others markers as EG103	See above
EG133 (*sod1*Δ*sod2*Δ)	*sod1::URA3 sod2*::*TRP1* double mutant, all others markers as EG103	See above
EG223 (*ctt1*Δ)	*ctt1::TRP1* all others markers as EG103	See above
BER/NER (WT)	*MATα ade2-101 his3*Δ*200 ura3*ΔNco lys2ΔBgl	Swanson *et al.* (1999)
Base excision repair mutants	Same genotype as BER/NER WT but *ntg1*Δ, *ntg2*Δ, *ntg1*Δ*ntg2*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ, *ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ	See above
W303 (WT)	*MAT***a** *ade2-1 leu2-3, 112 his3-11,15 trp1-1 ura3-1 can1-100*	A. Tzagoloff, New York
Mitochondrial mutants	Same genotype as W303 but *cox14*Δ, *cox15*Δ, *cox16*Δ, *cox17*Δ, *cox18*Δ, *cox20*Δ, *shy1*Δ, *sco1*Δ, *pet100*Δ, *pet117*Δ	See above

dilution in PB and plating (Pungartnik *et al.* 2005). Cells were plated on YPD and survival was determined after 3 d at 30 °C. Presented results are the mean of at least three independent experiments, the standard deviation and statistical analyses were calculated by GraphPad Prism® program.

Spontaneous mutation of mitochondrial mutants

STAT cells were grown in YPD at 30 °C for 72 h, washed twice with saline (0.9% NaCl, pH 5.0) and yeast suspensions plated on media SC (survival) and SC-Trp (spontaneous mutation). Following incubation for 7 d at 30 °C, colonies appearing on SC medium yielded data on cell survival, while those grown on SC-Trp represented the spontaneous mutations. Frequencies of spontaneous genomic mutation in different mitochondrial mutants (deficient of functional CcO) were scored per 10^7 cells. Results are means of three independent experiments, the standard deviation and statistical analyses was performed using the GraphPad Prism® software.

Results and discussion

Repair of Sn^{2+}-induced DNA lesions

The sensitivity of 35 different yeast strains (STAT cells of WT and isogenic mutants) to 60 min exposure at 25 mM of $SnCl_2$ is given in Tables 2 and 3. The range of killing of mutant cells as compared with the WT varied not more than 2 decades (survival between 1 and 90%, Table 2) or not at all (Table 3). Amongst the seven different DNA repair WT strains, survival varied from 25 to 90%, depending on the genetic background of each strain. Six of the WT strains can be roughly allocated to two sensitivity groups (survival either around 25 or 85%), with strain W303 in between (Table 2). These WT sensitivity variations were neutralized when comparing to sensitivities of

Table 2. Sensitivity to $SnCl_2$ (25 mM, 60 min) of STAT cells of different yeast strains.

Strains	$SnCl_2$	*p* value[a]	Relative sensitivity increase[b]
WT (XS2316)	**90.3 ± 3.3**		–
WT (XV185-14c)	**85.0 ± 8.0**		–
WT (W303)	**57.0 ± 5.4**		–
WT (Y10000)	**24.1 ± 1.1**		–
*rad2*Δ	13.5 ± 2.8	<0.05	1.4×
*rad4*Δ	10.3 ± 4.8	<0.01	1.5×
*rad6*Δ	8.1 ± 0.0	<0.0001	1.7×
*rad52*Δ	2.8 ± 0.9	<0.0001	2.5×
WT (YPH98)	**28.2 ± 6.2**		–
*snq1*Δ	14.5 ± 0.5	<0.05	1.5×
*snq2*Δ	23.0 ± 0.4	n.s.	1.1×
*snq3*Δ/*yap1*Δ	1.9 ± 0.2	<0.05	3.0×
WT (EG103)	**26.2 ± 6.2**		–
*sod1*Δ	5.3 ± 0.5	<0.01	2.5×
*sod2*Δ	17.5 ± 0.5	n.s.	1.4×
*sod1sod2*Δ	2.5 ± 0.2	<0.01	3.0×
*ctt1*Δ	49.0 ± 6.1	n.s.	0.6×
*ctt1*Δ*sod1*Δ	26.0 ± 2.3	n.s.	1.0×
WT	**87.2 ± 0.7**		–
*ntg1*Δ	77.4 ± 2.8	<0.01	1.7×
*ntg2*Δ	69.2 ± 2.2	<0.001	2.5×
*ntg1*Δ*ntg2*Δ	70.3 ± 1.6	=0.001	2.5×
*ntg1*Δ*ntg2*Δ*apn1*Δ	70.0 ± 3.4	=0.001	2.5×
*ntg1*Δ*ntg2*Δ*apn1*Δ*rad52*Δ	55.0 ± 1.3	<0.0001	4.0×
*ntg1*Δ*ntg2*Δ*apn1*Δ*rad1*Δ	31.3 ± 0.8	<0.0001	7.0×
*ntg1*Δ*ntg2*Δ*apn1*Δ*rev3*Δ	19.5 ± 0.6	<0.0001	11.0×

[a]Unpaired *t* test (95% interval confidence); statistical analyses comparing each mutant to its isogenic WT.[b]Sensitivity is defined by the inclination of an idealized linear inactivation curve in a semi-log plot. If WT survives 10% and mutant 1%, sensitivity increase of the mutant is by factor 2.n.s. not significantly different from the isogenic WT.

mutant strains, as always a set of WT and WT-derived isogenic mutant strains were compared. Sensitivities (or relative resistance) of mutant strains were calculated by comparison with the WT of semi-log graphs of respective survival curves.

It is known that $SnCl_2$ sensitivity in yeast increases from WT (RAD) < *rad2*Δ < *rad4*Δ < *rad6*Δ < *rad52*Δ in the Y10000 (EUROSCARF) background (Pungartnik *et al*. 2005). The recombination repair-deficient mutant *rad52*Δ had a 2.5-fold higher sensitivity as compared to the WT (Table 2). The relative resistance of mutant strains *rad2* and *rad4*, deficient in nucleotide excision repair (NER) was rather high, indicating a minor but significant contribution to repair of Sn^{2+} -induced DNA lesions by this repair pathway.

A series of mutants defective in different base excision repair (BER) pathways, combined with nucleotide excision repair (BER/NER, constructed elsewhere) were used to indirectly determine the type of $SnCl_2$-produced DNA lesion. Three DNA *N*-glycosylases, encoded by yeast genes *NTG1, NTG2*, and *OGG1* are known to be involved in repair of oxidative DNA damage that results in abasic sites in DNA (You *et al*. 1999, Alseth *et al*. 1999, Boiteux and Guillet 2004). Mutant allele *ntg2*Δ conferred the highest sensitivity (not to be enhanced in the *ntg1*Δ*ntg2*Δ*apn1*Δ triple mutant) demonstrating the necessity of the nucleus-located Ntg2p (Alseth *et al*. 1999) for repair of Sn^{2+} -induced DNA lesions (Table 2), whereas the apurinic site endonuclease Ntg1p, mainly localized in the mitochondria (You *et al*. 1999) and the

Table 3. Mutant alleles not affecting Sn^{2+}-resistance (25 mM, 60 min) of STAT cells.

Strains
q1 WT
q2* *gsh1ΔLWG1*
q3 *gsh1Δlwg1*
q4 *GSH1 lwg1*
*erg3*Δ [EUROSCARF]
*ogg1*Δ [EUROSCARF]
*mag1*Δ [EUROSCARF]
*gsh1*Δ* [EUROSCARF]
*ctt1*Δ [EUROSCARF]

*Grown in SynCo media supplemented with 100 μg GSH/ml.

OGG1-encoded *N*-glycosylase seem dispensable for repair of Sn^{2+}-induced DNA lesions (Tables 2 and 3).

Although all mutants containing *ntg2*Δ in conjunction with mutant alleles of other repair pathways (NER, error-prone or recombinational repair) had statistically significant higher sensitivity than the WT, the *rev3*Δ mutant allele-containing quadruple mutant strain was the most sensitive, thus indicating that the error-prone repair pathway (translesion synthesis (Lawrence 2002)) may make the highest contribution to repair of $SnCl_2$-induced DNA lesions (Table 2); this could explain the observed mutagenicity of $SnCl_2$ (Pungartnik *et al*. 2005). The response to $SnCl_2$-induced oxidative DNA damage thus differs from that introduced by hydrogen peroxide where the contribution of translesion synthesis is smaller than that of recombinational repair (Salmon *et al*. 2004). Introduction of a *rad52*Δ mutant allele, conferring lack of recombinational repair, into the BER triple knockout mutant *ntg1*Δ*ntg2*Δ*apn1*Δ led to a significant increase in sensitivity, demonstrating that the two repair modes contribute (at least in part) independently to removal of $SnCl_2$-induced DNA lesions (Table 2). The same can be said after the introduction of a *rad1*Δ mutant allele that yields an even more sensitive quadruple mutant strain. This indicates that NER, independently from BER or recombinational repair, can remove part of Sn^{2+}-induced DNA damage, most probably abasic sites (Torres-Ramos *et al*. 2000). These overlapping specificities of BER, NER, recombination and error-prone translesion synthesis in repair of damaged bases has already been shown by Swanson *et al*. (1999). Since DNA repair mechanisms were largely conserved during evolution (Eisen & Hanawalt 1999), the repair of Sn^{2+}-induced DNA damage via several different repair pathways in yeast might suggest a similar repair scenario in humans.

Membrane transport proteins influence Sn^{2+} toxicity

Two types of yeast membrane transporters were tested for their putative contribution to Sn^{2+}-uptake/homeostasis. Cells deficient in the facilitated transporter Atr1p/Snq1p (Kanazawa *et al*. 1988, Gömpel-Klein & Brendel 1990) showed significantly increased Sn^{2+}-sensitivity (factor 1.5) while a deletion mutant of the ABC transport protein Snq2p that so far has been shown to mediate resistance to structurally unrelated chemicals like 4-Nitro-quinoline oxide, sulphometuron methyl, triaziquone, and phenanthroline (Servos *et al*. 1993) was practically as resistant as the WT (resistance ranking was WT ≥ *snq2*Δ > *snq1*Δ). Complexity of metal ion homeostasis (Van Ho *et al*. 2002) however, makes it highly likely that other, hitherto unknown transport protein are also involved in Sn^{2+} transport (import/export). Indeed, it has been shown that $SnCl_2$ facilitates the Ca^{2+} entry through the L-type calcium channel under the condition of the membrane depolarization. There is the possibility that Ca^{2+} release from intracellular Ca^{2+} pools is involved in the action of $SnCl_2$ (Hattori *et al*. 2001) and that tin induces considerable changes in the metabolism of endogenous metals such as zinc and copper (Chmielnicka *et al*. 1981).

Lack of adaptive response to oxidative stress leads to Sn^{2+}-sensitivity

Mutants lacking yeast transcription factor Yap1p displayed a 3-fold higher Sn^{2+}-sensitivity than the isogenic WT (Table 2). Under oxidative stress Yap1 is oxidized and rapidly accumulated in the nucleus where it regulates the expression of up to 70 genes encoding proteins involved in oxidative stress response (Wood *et al*. 2004). Thus the yap1 mutant's sensitivity response indicates that anti-ROS defence systems of WT yeast are transcriptionally activated after Sn^{2+}-exposure. A similar response to oxidative stress exists in bacteria where the transcription activator OxyR induces the genes coding for

anti-stress proteins. Bacterial strains lacking a functional OxyR gene are used in the Mutoxitest to detect, via their specific sensitivity phenotype, ROS producing chemicals (Martínez *et al*. 2000). Such mutants also display a significantly higher sensitivity to Sn^{2+} (Pungartnik *et al*. 2005).

Yeast mutants lacking one or two genes encoding anti-oxidative defences (superoxide dismutase mutants *sod1*Δ, *sod2*Δ, and the double mutant *sod1*Δ*sod2*Δ) revealed 2–3-fold higher sensitivity to $SnCl_2$. The yeast strain containing both *sod* mutant alleles exhibited about additive sensitivities of the respective single mutants (Table 2). Judged by the higher sensitivity of *sod1*Δ mutant (Pungartnik *et al*. 2005), cytosolic Sod1p seems more important than mitochondrial Sod2p in protecting against the toxic effects of Sn^{2+} in STAT cells. On the other hand cytoplasmic catalase Ctt1p (Hartig & Ruis 1986) is apparently not involved in detoxification of any Sn^{2+}-induced ROS. In bacteria, H_2O_2 induces a cross-adaptive response to ROS-producing agents, amongst them $SnCl_2$ (Assis *et al*. 2002) suggesting that the OxyR transcription activator, which induces expression of catalase, alkyl hydroperoxide reductase and superoxide dismutase protects against Sn^{2+}-generated oxidative stress. The Yap1 transcription activator in yeast may have the same function (Wu & Mowe-Rowley 1994). However, this protective response to oxidative stress does not render a yeast cell about 1,000-fold more resistant (as calculated in exposure dose necessary for a like-wise killing) to Sn^{2+} as is seen when changing from glucose-repressed LOG to the glucose de-repressed STAT phase (cf. below and Figures 3 and 4). It has been suggested that two independently acting anti-ROS protective systems (one mediated by glucose repression/de-repression, the other via ROS-inducible transcription activators) are working in yeast (Maris *et al*. 2001), and our data imply that both are contributing to Sn^{2+}-resistance.

Defects in respiratory chain lead to Sn^{2+}-sensitivity

Yeast strains containing mutant alleles of genes encoding proteins of mitochondrially located cytochrome c oxidase (CcO-deficient mutants), also showed enhanced and variable sensitivity to

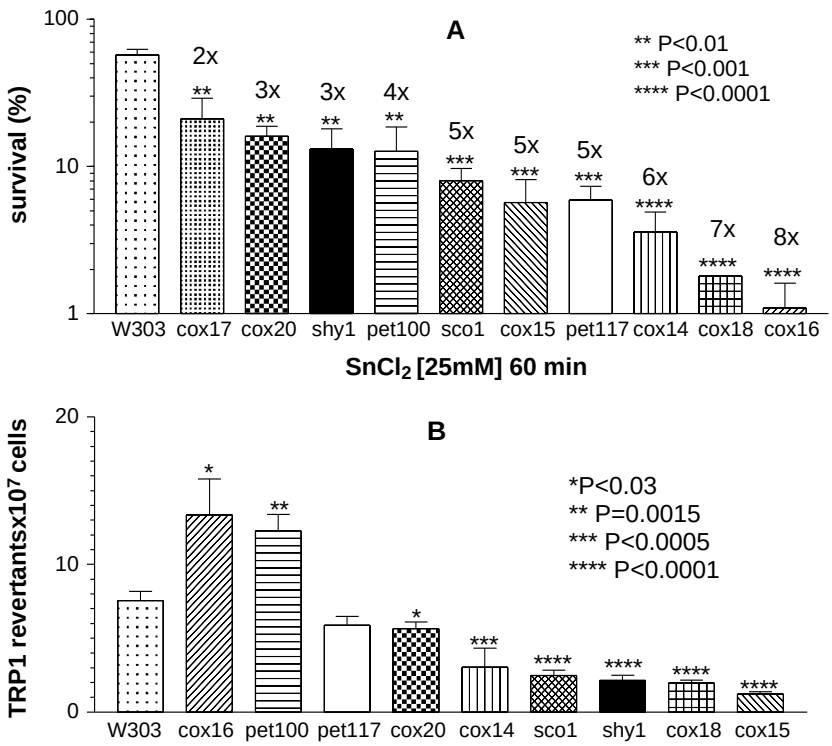


Figure 1. (A) Survival of STAT cells of WT and 9 mitochondrial mutants after 60 min $SnCl_2$ exposure (25 mM). (B) Reversion of tryptophan mutant allele *trp1-1* in mitochondrial mutant strains *(trp1-1)*, per 10^7 survivors. Numbers above the error bar of each column (A) gives the sensitivity increase of the respective mutant. Cells were diluted in PB.

$SnCl_2$ (Figure 1A). Interestingly, this sensitivity could be strongly enhanced in a *cox11/pso7-1* mutant (Pungartnik *et al.* 1999) when introducing the *erg3/pso6* mutant allele (Schmidt *et al.* 1999), as a second mutation (Brendel *et al.* 2003). Alone, the *erg3/pso6* mutant allele confers ergosterol deficiency and renders the mutant not sensitive to Sn^{2+} (Table 3), suggesting a non-protective role of this membrane constituent against the oxidative stress induced by this metal. In combination with *cox11/pso7-1* mutant allele, however, *erg3/pso6* showed a dramatic sensitivity effect. Generally CcO-deficient mutants are thought to produce more H_2O_2 by letting electrons escape from the respiratory chain (Barros *et al.* 2003). This elevated H_2O_2 might act as a mutagen on genomic DNA. We assayed, therefore, for spontaneously induced mutations in the *trp1-1* locus (reversion to trp^+) in mutant alleles of 9 different CcO-encoding genes (Figure 1B). While 2 CcO mutants, *cox16* and *pet100* had indeed higher-than-WT mutability in *trp1-1*, six others, i.e., the majority showed lower-than-WT mutability (Figure 1B), so that a general assumption of higher spontaneous mutation in CcO mutants could not be verified. It is known that Sn^{2+} generates ROS via Fenton-like reactions (McLean *et al.* 1983) and that there is variable content of ROS being produced in different CcO mutants (Barros *et al.* 2003). This might be the reason for, or at least contribute to, the observed variation of Sn^{2+}-sensitivity in the CcO mutants (Figure 1A). It must be emphasized, however, that total lack of respiratory chain activity in rho^0 mutants does not lead to enhanced Sn^{2+}-sensitivity, as isogenic rho^+ and rho^0 strains (YPH98 and q1, respectively) have identical WT-like survival (Tables 2 and 3).

Diauxic shift-induced Sn^{2+}-resistance

The highest $SnCl_2$ -sensitivity, however, was observed in glucose-repressed pre-diauxic shift exponentially growing cells (LOG cells (Figure 2)). On the basis of comparison to the $SnCl_2$ exposure dose required for likewise inactivation of STAT cells we found a more than 1000-fold increase in sensitivity (LOG slightly more sensitive to 25μM Sn^{2+} than STAT at 25 mM (Figure 3)). The significant increase of sensitivity of sod mutants as compared to the WT was the same in LOG cells at 25 μM Sn^{2+} exposure as seen in STAT cells at the

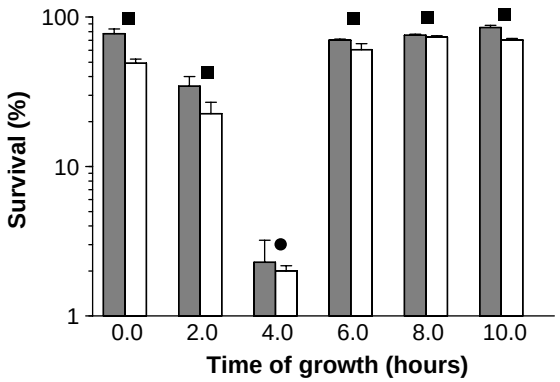


Figure 2. Sensitivity to 20 minutes Sn^{2+}-exposure of haploid WT strain Y10000 (grey column) and *rad52*Δ (white column); (■) STAT cells exposed to 25 mM; (●) LOG cells exposed to 2.5 mM. Cells were diluted in PB.

1000-fold Sn^{2+} exposure dose (Table 2). The high resistance to Sn^{2+} is acquired during and after the diauxic shift, i.e., when the yeast LOG cells are released from glucose repression and many cellular functions are adapted to respiratory metabolism. This resembles the response of yeast cells during diauxic shift-induced resistance against hydroperoxides (Maris *et al.* 2001). This process is independent of one tested repair function (Rad52p) (Figure 2), of the presence of superoxide dismutases (Figure 3), functional cytochrome c oxidase (Figure 4), and the presence of any mitochondrial respiratory metabolism (rho^0 mutants).

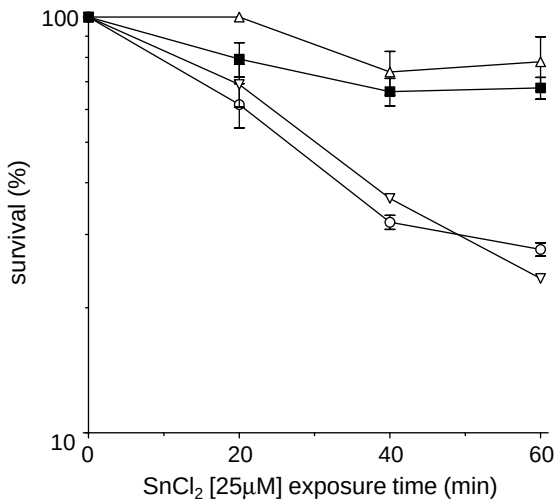


Figure 3. Sensitivity to 25 μM Sn^{2+}-exposure of LOG cells of haploid WT strain EG103 (■); and its isogenic mutants *sod2*Δ (△); *sod1*Δ (▽); and the double mutant *sod1*Δ*sod2*Δ (○).

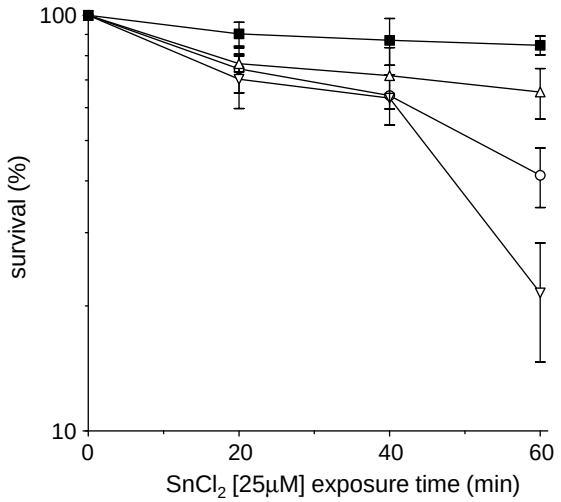


Figure 4. Sensitivity to 25 μM Sn^{2+}-exposure of LOG cells with different defects in cytochrome *c* oxidase WT W303 (■); *cox15*Δ (□); *sco1*Δ (○) and *cox17*Δ (▽).

Glutathione not needed to protect against short-term Sn^{2+}-exposure

Interestingly, mutants with low and extremely low glutathione (GSH) pools showed no enhanced sensitivity to Sn^{2+} (Table 3). Mutant strain q2 (*gsh1*Δ) that lacks the first step of the two-step GSH biosynthesis and relies totally on externally offered (low) GSH showed the same Sn^{2+}-resistance as the q1 WT. The same was true for mutant q3 which is isogenic with mutant strain q2 but, due to a mutational change in the second enzyme of the proline biosynthetic pathway produces a little amount of the dipeptide γ-glutamylcysteine (the product lacking in the *gsh1* mutant (Spector *et al.* 2001)), and hence GSH, and is thus independent of external GSH supplementation. Finally mutant q4 that is WT for GSH biosynthesis and contains only the altered enzyme of the proline pathway, was also WT-like in its Sn^{2+}-resistance phenotype. Thus, GSH is not needed for the protection of yeast against acute, short-term (i.e. 1 h) Sn^{2+}-exposure (Table 3), most probably because catalase provides an overlapping defence system against metal-induced ROS (Grant *et al.* 1998).

The combined action of all above-mentioned protective mechanisms, whose functions were shown in STAT cells, i.e. DNA repair, membrane transport, and defences against ROS, can hardly explain the difference of three orders of magnitude in $SnCl_2$-sensitivity between isogenic LOG and STAT cells. Actually, some protective mechanisms, e.g., Sod1 and Sod2 as well as functionality of cytochrome c oxidase may be totally discounted in this comparison, as the *sod1* and *sod2* mutant alleles (Figure 3) as well as CcO mutants (Figure 4) conferred enhanced $SnCl_2$-sensitivity in STAT and LOG cells alike, regardless of their 1000-fold difference in sensitivity. Thus, this extreme sensitivity of LOG cells (alternatively, the extreme resistance of STAT cells (De Winde *et al.* 1997)) either suggests that cells growing under glucose repression lack at least one, most probably several, unknown mechanism(s) protecting against ROS or other stress (Fuge & Werner-Washburne 1997) or that the protection factors already known to us (c.f. above) have an extremely synergistic interaction (i.e. overlapping functionality) in STAT cells; this would not show us the real protective potential of a single metabolic contribution (as is suggested for the contribution of different repair mechanisms to removal of Sn^{2+}-induced DNA lesions (cf. above and Swanson *et al.* 1999)), but a joint suppression of several of these protective mechanisms (e.g. under glucose repression) would render a LOG cell extremely sensitive. Alternatively or additionally, we might speculate that the high sensitivity of LOG cells could also be, at least partially, due to a more efficient uptake of Sn^{2+} ions as rapidly growing cells might have a more active membrane transport. One step towards clarifying this last question might be the quantitative determination of Sn^{2+}-uptake by molecular dosimetry methods, e.g. via PIXE (particle induced X-ray emission) in isogenic LOG and STAT cells (Viau *et al.* in press). This would also allow us to better assess the genotoxic potential of intracellular Sn^{2+} at different physiological states of the yeast cell. Clearly, there is need for clarifying the types of ROS being directly or indirectly formed by Sn^{2+}, and more information on this may be gained by studying the response of all yeast strains known to have a defect in anti-ROS defence (single or multiple allele mutants) and by complementing this info by *in vitro* biochemical studies.

Despite of the LOG/STAT cells Sn^{2+}-sensitivity/resistance riddle we may summarize our results to partially answer two questions: (1) What type of DNA damage is induced by Sn^{2+}? We know that strand breaks are formed *in vitro* (Dantas *et al.* 1999), and this would best explain the contribution

of recombinational repair; oxidized base damage would explain the necessity of BER repair; some bulky adducts could explain involvement of NER in repair; finally, and most important, translesion synthesis would allow resumption of DNA synthesis at stalled replication forks, at the cost of error-prone repair (mutation). (2) Which species of ROS are generated by intracellular Sn^{2+}? Clearly superoxide anion, as Sod1p, and to a lesser extent, Sod2p are protecting the cells; hydrogen peroxide is most probably generated only in little quantity (or not at all) as cytosolic catalase and GSH are not necessary for protection. Direct base oxidation may occur as indicated by the role of BER in repair, but 8-hydroxyguanine, the specific substrate of Ogg1p, is apparently not formed.

Acknowledgements

We thank Dr. A. Tzagoloff for kindly providing the mitochondrial mutant strains and Dr. M. Grey for constructing the q mutants. Research supported by Fundação de Amparo a Pesquisa do Estado da Bahia (FAPESB), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and GENOTOX. C. P. held a fellowship by FAPESB/CNPq (PRODOC Program). M.B. is Visiting Scientist supported by FAPESB. Part of the data is from the Master thesis of C.V. (PPGBCM-UFRGS).

References

Alseth I, Eide L, Pirovano M, Rognes T, Seeberg E, Bjoras M. 1999 The *Saccharomyces cerevisiae* homologues of endonuclease III from *Escherichia coli*, Ntg1 and Ntg2, are both required for efficient repair of spontaneous and induced oxidative DNA damage in yeast. *Mol Cell Biol* **19**, 3779–3787.

Assis ML, de Mattos JC, Caceres MR, *et al*. 2002 Adaptive response to H_2O_2 protects against $SnCl_2$ damage: the OxyR system involvement. *Biochimie* **84**, 291–294.

Barros MH, Netto LES, Kowaltowski AJ. 2003 H_2O_2 generation in *Saccharomyces cerevisiae* respiratory *PET* mutants: effect of cytochrome C. *Free Rad Biol Med* **35**, 179–188.

Bernardo-Filho M, Cunha MC, Valsa JO, Caldeira-de-Araújo A, Silva FCP, Fonseca AS. 1994 Evaluation of potential genotoxicity of stannous chloride: inactivation, filamentation and lysogenic induction of *Escherichia coli*. *Food Chem Toxicol* **32**, 477–479.

Boiteux S, Guillet M. 2004 Abasic sites in DNA: repair and biological consequences in *Saccharomyces cerevisiae*. *DNA Repair* **3**, 1–12.

Brendel M, Revers LF, Strauss M, *et al*. 2003 The role of PSO genes in repair of DNA damage of *Saccharomyces cerevisiae*. *Mutat Res* **544**, 179–193.

Burke D, Dawson D, Stearns T. 2000 Methods in yeast genetics. N.Y.: Cold Spring Harbour Laboratory Course Manual, CSH Laboratory Press.

Chmielnicka J, Szymanska JA, Sniec J. 1981 Distribution of tin in the rat and disturbances in the metabolism of zinc and copper due to repeated exposure to $SnCl_2$. *Arch Toxicol* **47**, 263–268.

Dantas FJ, Moraes MO, Carvalho EF, Valsa JO, Bernardo-Filho M, Caldeira-de-Araujo A. 1996 Lethality induced by stannous chloride on *Escherichia coli* AB1157: participation of reactive oxygen species. *Food Chem Toxicol* **34**, 959–962.

Dantas FJ, Moraes MO, de Mattos JC, *et al*. 1999 Stannous chloride mediates single strand breaks in plasmid DNA through reactive oxygen species formation. *Toxicol Lett* **110**, 129–136.

De Winde JH, Thevelein JM, Winderickx J. 1997 From feast to famine: adaptation to nutrient depletion in yeast. In: Hohmann S, Mager WH, eds *Yeast Stress Responses*. Berlin, Heidelberg, New York: Springer, pp. 7–52.

Eide D, Guerinot ML. 1997 Metal ion uptake in eukaryotes: research on *Saccharomyces cerevisiae* reveals complexity and insights about other species. *ASM News* **63**, 199–205.

Eisen JA, Hanawalt PC. 1999 A phylogenomic study of DNA repair genes, proteins and processes. *Mutat Res* **435**, 171–213.

Fuge EK, Werner-Washburne M. 1997 Stationary phase in the yeast *Saccharomyces cerevisiae*. In: Hohmann S, Mager WH, eds *Yeast Stress Responses*. Berlin, Heidelberg, New York: Springer, pp. 53–74.

Gömpel-Klein P, Brendel M. 1990 Allelism of *SNQ1* and *ATR1*, genes of the yeast *Saccharomyces cerevisiae* required for controlling sensitivity to 4-nitroquinoline-*N*-oxide and aminotriazole. *Curr Genet* **18**, 93–96.

Grant CM, Perrone G, Dawes IW. 1998 Glutathione and catalase provide overlapping defenses for protection against hydrogen peroxide in the yeast *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun* **253**, 893–898.

Hartig A, Ruis H. 1986 Nucleotide sequence of the *Saccharomyces cerevisiae CTT1* gene and deduced amino-acid sequence of yeast catalase T. *Eur J Biochem* **160**, 487–490.

Hattori T, Maehashi H, Miyazawa T, Naito M. 2001 Potentiation by stannous chloride of calcium entry into osteoblastic MC3T3-E1 cells through voltage-dependent L-type calcium channels. *Cell Calcium* **30**, 67–72.

Heuchel R, Radtke F, Georgiev O, Stark G, Aguet M, Schaffner W. 1994 The transcription factor MTF-1 is essential for basal and heavy metal-induced metallothionein gene expression. *EMBO J* **13**, 2870–2875.

Kanazawa S, Driscoll M, Struhl K. 1988 *ATR1*, a *Saccharomyces cerevisiae* gene encoding a transmembrane protein required for aminotriazole resistance. *Mol Cell Biol* **8**, 664–673.

Lawrence CW. 2002 Cellular roles of DNA polymerase ζ and Rev1 protein. *DNA Repair* **1**, 425–435.

Machida I, Nakai S. 1980 Induction of spontaneous and UV-induced mutations during commitment to meiosis in *Saccharomyces cerevisiae*. *Mutat Res* **73**, 59–68.

Maris AF, Assumpção ALK, Bonatto D, Brendel M, Henriques JAP. 2001 Diauxic shift-induced stress resistance against hydroperoxides in *Saccharomyces cerevisiae* is not an

adaptive stress response and does not depend on functional mitochondria. *Curr Genet* **39**, 137–149.

Martínez A, Urios A, Blanco M. 2000 Mutagenicity of 80 chemicals in *Escherichia coli* tester strains IC203, deficient in OxyR, and its oxyR(+) parent WP2 uvrA/pKM101: detection of 31 oxidative mutagens. *Mutat Res* **467**, 41–53.

McLean JR, Kaplan JG. 1979 The effect of tin on unscheduled and semi-conservative DNA synthesis. In: Kaplan JG, ed *The Molecular Basis of Immune Cell Function*. Amsterdam: Elsevier Biomedical.

McLean JR, Blakey DH, Douglas GR, Kaplan JR. 1983a The effect of stannous and stannic (tin) chloride on DNA in Chinese hamster ovary cells. *Mutat Res* **119**, 195–201.

McLean JR, Birnboim HC, Pontefact R, Kaplan JG. 1983 The effect of tin chloride on the structure and function of DNA in human white blood cells. *Chem Biol Interac* **46**, 189–200.

McMurray CT, Tainer JA. 2003 Cancer, cadmium and genome integrity. *Nat Genet* **34**, 34239–34241.

Pungartnik C, Viau C, Picada J, Caldeira-de-Araújo A, Henriques JAP, Brendel M. 2005 Genotoxicity of stannous chloride in yeast and bacteria. *Mutat Res* **583**, 146–157.

Pungartnik C, Kern MF, Brendel M, Henriques JAP. 1999 Mutant allele *pso7-1*, that sensitizes *Saccharomyces cerevisiae* to photoactivated psoralen, is allelic with *COX11*, encoding a protein indispensable for a functional cytochrome c oxidase. *Curr Genet* **36**, 124–129.

Salmon TB, Evert BA, Song B, Doetsch PW. 2004 Biological consequences of oxidative stress-induced DNA damage in *Saccharomyces cerevisiae*. *Nucl Acid Res* **32**, 3712–3723.

Schmidt CL, Grey M, Schmidt M, Brendel M, Henriques JAP. 1999 Allelism of *Saccharomyces cerevisiae* genes *PSO6*, involved in survival after 3-CPs + UVA induced damage, and *ERG3*, encoding the enzyme sterol C-5 desaturase. *Yeast* **15**, 1503–1510.

Schroeder HA, Balassa JJ, Tipton IH. 1964 Abnormal trace metals in man: tin. *J Chron Dis* **17**, 483–502.

Servos J, Haase E, Brendel M. 1993 Gene*SNQ2* of *Saccharomyces cerevisiae*, which confers resistance to 4-nitroquinoline-*N*-oxide and other chemicals, encodes a 169 kDa protein homologous to ATP-dependent permeases. *Mol Gen Genet* **236**, 214–218.

Spector D, Labarre J, Toledano MB. 2001 A genetic investigation on the essential role of glutathione: mutations in the proline biosynthetic pathway are the only suppressors of glutathione auxotrophy in yeast. *J Biol Chem* **276**, 7011–7016.

Swanson RL, Morey NJ, Doetsch PW, Jinks-Robertson S. 1999 Overlapping specificities of base excision repair, nucleotide excision repair, recombination, and translesion synthesis pathway for DNA base damage in *Saccharomyces cerevisiae*. *Mol Cell Biol* **19**, 2929–2935.

Tomsett AB, Thurmann DA. 1988 Molecular biology of metal tolerance of plants. *Plant Cell Environ* **11**, 383–394.

Torres-Ramos CA, Johnson RE, Prakash L, Prakash S. 2000 Evidence for the involvement of nucleotide excision repair in the removal of abasic sites in yeast. *Mol Cell Biol* **20**, 3522–3528.

Van Ho A, McVery Ward D, Kaplan J. 2002 Transition metal transport in yeast. *Annu Rev Microbiol* **56**, 237–261.

Viau CM, Yoneama ML, Dias JF, Pungartnik C, Brendel M, Henriques JAP (2005) Detection and quantitative determination by PIXE of the mutagen Sn^{2+} in yeast cells. *Nucl. Instrum Meth Physics Res B*, in press.

von Borstel RC, Cain KT, Steinberg CM. 1971 Inheritance of spontaneous mutability in yeast. *Genetics* **69**, 17–27.

Wehner EP, Rao E, Brendel M. 1983 Molecular structure and genetic regulation of *SFA*, a gene responsible for resistance to formaldehyde in *Saccharomyces cerevisiae*. *Mol Gen Genet* **237**, 351–358.

Wood MJ, Storz G, Tjandra N. 2004 Structural basis for redox regulation of Yap1 transcription factor localization. *Nature* **430**, 917–921.

Wu A, Moye-Rowley WS. 1994 *GSH1*, which encodes gamma-glutamylcysteine synthetase, is a target gene for yAP-1 transcriptional regulation. *Mol Cell Biol* **14**, 5832–5839.

You HJ, Swanson RL, Harrington C, *et al.* 1999 *Saccharomyces cerevisiae* Ntg1p and Ntg2p: broad specificity *N*-glycosylases for the repair of oxidative DNA damage in the nucleus and mitochondria. *Biochemistry* **38**, 11298–11306.

Testes preliminares de sensibilidade do cacaueiro (*Theobroma cacao*) ao agente químico cloreto de estanho ($SnCl_2$)

1ª Tentativa:

1. As plantas de *T. cacao* foram regadas por 45 dias com o $SnCl_2$ nas concentrações de 5, 25 e 50 µM;
2. Foram testadas 6 plantas como controle e 6 plantas para cada dose utilizada;
3. Nenhuma das plantas tratadas apresentou sensibilidade ao $SnCl_2$.

2ª Tentativa:

1. As plantas de *T. cacao* foram regadas por 45 dias com o $SnCl_2$ nas concentrações de 150, 300 e 600 µM;
2. Foram testadas 6 plantas como controle (Figura 1A) e 6 plantas para cada dose utilizada;
3. Nas duas primeiras doses, nada aconteceu às plantas (Figuras 1B E 1C);
4. Na última dose, as plantas apresentaram sensibilidade ao $SnCl_2$ (Figura 1D).

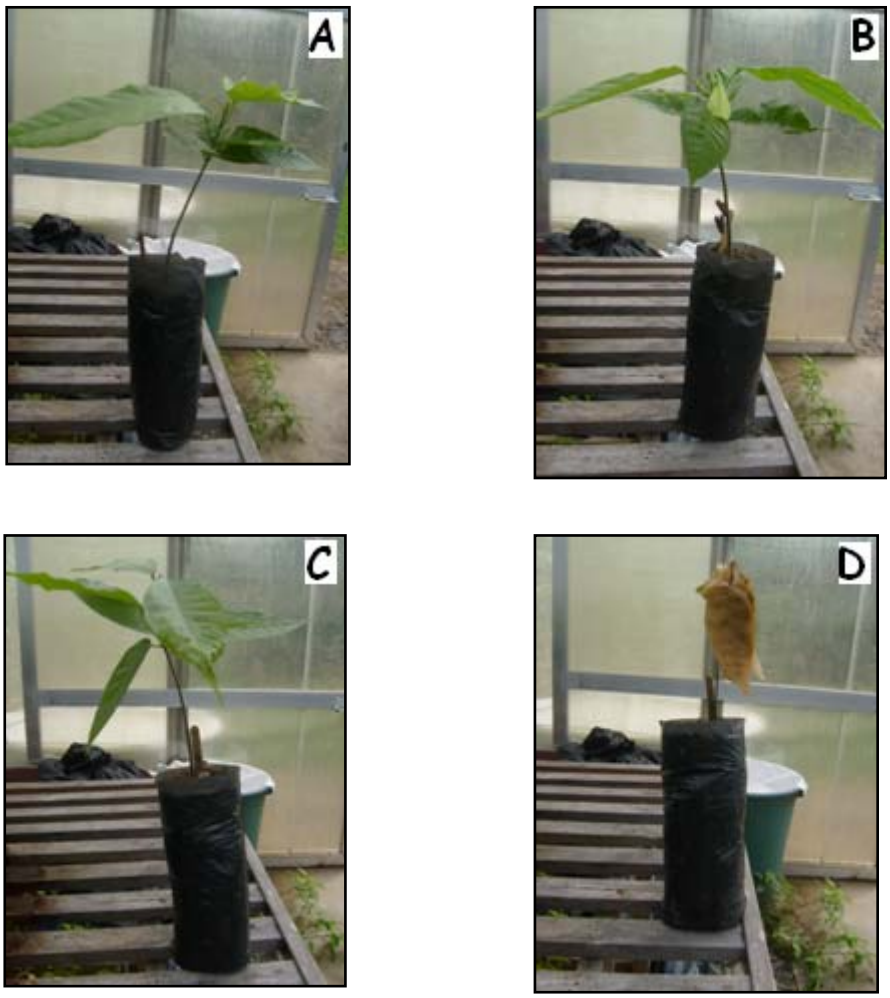


Figura 1. Plantas de *Theobroma cacao* tratadas com $SnCl_2$. (A) controle positivo; (B) tratada com 150 µM; (C) tratada com 300 µM; (D) tratada com 600 µM de $SnCl_2$.