Ultraviolet and microwave radiation in *Trichoderma viride* isolates

Neiva Braun, Lilian Castilho dos Santos,
Solange Bosio Tedesco, Juçara Teresinha Paranhos,
Antonio Carlos Ferreira da Silva

Departamento de Biologia/CCNE/UFSM - Santa Maria, RS
e-mail: acfsilva@smail.ufsm.br

Abstract

This paper aimed to determine the exposition time close to 5% of survival of *Trichoderma viride* (TSM) under ultraviolet light, and TSM and IT isolates of *Trichoderma viride* to microwave radiation, in order to obtain the mutants resistant to benomyl fungicide. This survival was studied from the irradiation of conidia at different exposition times. 0.1mL aliquots of conidia suspension from the isolates were irradiated at time of 5% of survival and incubated in PDA medium supplemented with doses of 0; 1.0; 5.0; and 10 $\mu g.mL^{-1}$ of benomyl. The results showed that the exposition time of the TSM isolate to ultraviolet radiation was about two minutes and for microwave radiation the time was eight seconds for both TSM and IT isolates. Conidia from IT isolate were more resistant to microwave radiation than the TSM isolate. Eight resistant mutants to 5.0 $\mu g.mL^{-1}$ benomyl fungicide were obtained from the TSM isolate exposed to ultraviolet radiation, both according to the resistance to the fungicide and the antagonism *in vitro* to phytopathogens, though with different colors of mycelia. Colonies of mutants resistant to benomyl with the use of microwave radiation were not observed.

Key words: mutants, radiation, *Trichoderma*, benomyl.

Resumo

Este trabalho teve como objetivo determinar o tempo de exposição próximo a 5% de sobrevivência do isolado *Trichoderma viride* (TSM) à radiação ultravioleta e dos isolados TSM e IT de *T. viride* à radiação micro-ondas, visando à obtenção de mutantes resistentes ao fungicida benomil. A sobrevivência do isolado TSM à luz ultravioleta e dos isolados TSM e IT à

radiação microondas foi estudada a partir da irradiação da suspensão de conídios em diferentes tempos de exposição. Alíquotas de 0,1 mL das suspensões de conídios dos isolados foram irradiadas no tempo de 5% de sobrevivência e incubadas em meio BDA suplementado com doses de 0; 1,0; 5,0 e 10 $\mu g.mL^{-1}$ de benomil. Os resultados mostraram que o tempo de exposição do isolado TSM à radiação ultravioleta para obtenção de aproximadamente 5% de sobrevivência foi de dois minutos, e para a radiação microondas este tempo foi de oito segundos em ambos isolados TSM e IT. Conídios do isolado IT foram mais resistentes à radiação microondas em comparação ao isolado TSM. Obteve-se a partir do isolado TSM exposto à radiação ultravioleta, 8 mutantes resistentes a 5,0 $\mu g.mL^{-1}$ do fungicida benomil estáveis quanto a resistência ao fungicida e antagonismo *in vitro* a fitopatógenos, mas apresentando diferenças na coloração de micélios. Não foram observadas colônias mutantes resistentes ao benomil com a utilização da radiação microondas.
Palavras-chave: mutantes, radiação, *Trichoderma*, benomil.

Introduction

Among biocontrol microorganisms which mycoparasite a range of phytopathogens, there are many species of *Trichoderma* sp. that have already been studied, presenting promising characteristics for the development of integrated chemical and biological controls as an efficient alternative to reduce the use of chemical products (SILVA, 1991; SILVA, 1997). However, in order to make the control work the induction of resistance in the biocontrol microorganism and the consequent selection of stable mutants is necessary, so that it may be used simultaneously with the chemical control in reduced doses. The obtention of these resistant antagonists turned out to be possible through radiation, because mutagenesis induced by physical agents may produce mutants with stable features (PAPAVIZAS, 1980).

Both ultraviolet and microwave radiation, due to their efficiency, simplicity and safety of use in a laboratory, are recommended to induce mutants. Therefore, if there is the need for a fungus which is resistant to a fungicide, such as benomyl for instance, the use of radiation in appropriate dosage and the transference of irradiated conidia to a medium containing fungicide makes the obtention of mutants possible (MELO, 1998). Before inducing the mutation, it is recommended that the survival of the fungus to the chosen mutagenic is studied (SILVA, 1997; PACCOLA-MEIRELLES, 1998). Therefore, the necessary dose for isolating mutant conidiamay be determined through the results of survival (SILVA, 1991).

The research reported in this paper aimed to provide information

about survival of TSM isolate of *Trichoderma viride* under ultraviolet light and TSM and IT isolates of *Trichoderma viride* to microwave radiation, in order to obtain mutants resistant to benomyl fungicide.

Material and methods

The experiments were carried out at the Laboratory of Microorganisms and Biological Control of Phytophatogens of the Department of Biology at the Federal University of Santa Maria (UFSM), Santa Maria, (RS), Brazil.

Trichoderma viride isolates and phytopathogens

TSM (SILVA, 1997) and IT isolates (from garden soil/ Santa Maria/(RS)) of *Trichoderma viride*, sensitive at concentrations above 1.0 $\mu g.mL^{-1}$ of benomyl fungicide and antagonists to *Sclerotinia sclerotiorum* (SILVA, 1997) were provided by the Laboratory of Microorganisms and Biological Control of Phytophatogens of the Department of Biology of the Exact and Natural Sciences Center at the Federal University of Santa Maria (UFSM).

The phytopathogens *Sclerotinia sclerotiorum*, *Fusarium* spp. and *Rhizoctonia solani*, used in the dual culture, were provided by the Laboratory of Phytophatology of the Rural Sciences Center at UFSM, (RS). The mycelia and spores of the antagonist isolate and phytopathogens were kept at temperature close to 10° C in disks of PDA medium (potato-dextrose agar) in aseptic recipients with 5mL of sterilized distillated water without light until their uses.

Survival to ultraviolet radiation

This essay aimed to study the survival of TSM isolate of *Trichoderma viride* to ultraviolet radiation and to determine the time of exposure to this agent for the dose of 5% of survival of conidia used as pattern for the mutant induction.

The isolate was incubated in PDA medium (potato-dextrose-agar) and, after seven days, the concentration of conidia suspended in Tween 80 solution was determined (CASSIOLATO & MELO, 1999) through the dilution technique (NEDER, 1992; FERNANDES, 1993). After that, 9mL of suspension with $1.8x10^3$ conidia mL^{-1} of TSM isolate were transferred to a Petri dish and sterilized. Next, the irradiation took place by using a dark chamber with ultraviolet light (40W, 256nm) at a distance of 12cm of the dish containing the suspension. Then, the Petri dishes were opened at 0,

15, 30, 45, 60, 75, 90, 120, 135 and 150 seconds. After exposure to radiation, 0.1mL of conidia suspension was transferred to dishes containing PDA culture medium with 0.1% sodium deoxycolate.

The incubation took place for 48 hours in the dark, the counting of surviving conidia was performed by means of observation of the number of colony number. From these data, the time of radiation close to 5% of survival was determined (SILVA, 1991; NEDER, 1992; CASSIOLATO, 1995).

Survival to the microwave agent

This essay aimed to study the survival of TSM and IT isolates of *Trichoderma viride* to microwave radiation and to determine the time of exposure to this agent for the dose of 5% of survival of conidia used as pattern for the mutant induction.

The isolates were incubated in PDA medium and, after seven days, the concentration of conidia was determined by using the same process of the essay for the survival to ultraviolet radiation. The exposition time to microwave radiation by using a household appliance (2450MHz, 900W, Consul CMU31A) were the following: 0, 4, 6, 8, 12, 16 and 20 seconds for both isolates. Each treatment was repeated five times. After 48 hours, the counting of surviving conidia was performed. From these data, the time of radiation close to 5% of survival was determined.

Obtaintion of mutants resistant to benomyl fungicide

Aiming to obtain mutants resistant to benomyl fungicide, conidia in suspension of wild TSM isolate of *T. viride*, sensitive to doses higher than 1.0 $\mu g.mL^{-1}$ of benomyl fungicide, were exposed to ultraviolet light and microwave radiation at times of 5% survival.

The solution of benomyl fungicide [1-(butycarbamoyl) -2-benzimidazole carbamic acid] in the formulation of Benlate 500 (50% active ingredient, Du Pont do Brasil S. A.) was prepared by the Edington *et al.* (1971), modified by MENTEN *et al.* (1976), by suspending it in 5.0mL acetone and completing the volume up to 100mL of sterilized distilled water (solutions). The dilutions were made according to SILVA *et al.* (1999).

Aliquots of 0.1mL of the irradiated suspensions were transferred to PDA medium supplemented with benomyl (active ingredient) at concentrations of 0 (control concentration); 1.0; 5.0 and 10 $\mu g.mL^{-1}$, for the essays with microwave and ultraviolet radiation. In the control treatment, the suspension of wild TSM isolate was not exposed to irradiation, being directly transferred to PDA medium without fungicide. Subsequently, counts of the surviving conidia were performed. Each

treatment was repeated five times. Analysis of variance was applied, and the means were compared by Tukey test, 5% probability.

Stability of resistance test

In order to test the stability of the resistance of benomyl, mutants of *Trichoderma viride* resistant to 5.0 μg.mL^{-1} of benomyl fungicide were transferred three times every seven days consecutively to fungicide-free PDA medium, and then transferred back to PDA medium at 5.0 μg.mL^{-1} concentration of benomyl fungicide. After three weeks, morphological alterations in of the mutant colonies were evaluated, mainly the color of the mycelia. So as to test the stability of the antagonism to the phytopathogens *Sclerotinia scletotiorum*, *Fusarium* spp. and *Rhizoctonia solani in vitro*, the evaluation of the mutants was performed through direct observation (BELL *et al*., 1982; SILVA, 1997; ETHUR *et al*., 2005), in which the disks of the pathogen and the antagonist are placed in opposite sides of a Petri dish in order to show the antagonism. After seven days, in a chamber at 25° C and photoperiod of 12 hours, the evaluation was carried out according to the criteria proposed by BELL *et al*. (1982), modified by ETHUR *et al.* (2001), in which the following grading scale is used: 1 (antagonist grows all over the Petri dish); 1.5 (antagonist grows over more than 2/3 of the dish); 2 (antagonist grows 2/3 over the dish); 2.5 (antagonist grows from 2/3 to ½ of the dish); 3 (antagonist and pathogen grow over half dish, none dominates the other); 4 (pathogen grows 2/3 over the dish) and 5 (pathogen grows all over Petri dish). The modification constitutes an increase of grades with median values of 1.5 and 2.5. Each treatment had five repetitions. Analysis of variance was performed and the means were compared by Tukey test, 5% probability.

Results and discussion

The results presented in Table 1 show that the increasing time of exposure of the spores of TSM isolate of *T. viride* to ultraviolet radiation leads to a decreasing number of surviving colonies.

For the TSM conidia suspension on the Petri dish (Table 1), a steep decreased in of the colony survival was observed, and conidia survival was less than 50% in the first 15 seconds of exposure.

The time closest to 5% survival was reached with two minutes of exposure (Table 1), reaching a mean of 4.59% of surviving colonies.

The results presented in Table 2 show that as exposure time to radiation increased the number of surviving colonies decreased for both the TSM and IT isolates.

Table 1. Number and percentage of TSM isolate colonies of *Trichoderma viride* surviving to ultraviolet radiation on Petri dish (mean of five repetitions).

Exposition time to ultraviolet radiation (s)	Number of surviving colonies	% of surviving colonies
0	123	100.00
15	34	27.64
30	22	17.88
45	17	13.82
60	13	10.57
75	11	8.94
90	8	6.50
120	6	4.87
135	5	4.06
150	3	2.43

Table 2. Number and percentage of colonies of TSM and IT isolates of *Trichoderma viride* surviving to microwave radiation (mean of five repetitions).

Time of radiation (s)	Number of colonies		% of surviving colonies	
	TSM	IT	TSM	IT_1
0	253	660	100.00	100.00
4	48	87	18.97	13.18
8	16	30	6.32	4.45
12	3	18	1.18	2.72
16	0	10	0.00	1.51
20	0	3	0.00	0.45

The IT isolate proved to be more resistant to microwave radiation, with surviving colonies at exposure times above 16 seconds, unlike what was observed for the TSM isolate.

The time closest to 5% survival was eight seconds of exposure for both isolates, and the percentage of survival was 4.45% and 6.32% for IT and TSM isolates, respectively.

The results presented in Table 3 are in agreement with what was mentioned above(Table 1) according to exposure time which causes 5% of survival, through comparison between the number of colonies without exposure to ultraviolet radiation in the control treatment (1004) and the number of colonies after exposition to radiation in treatment 0.0 (46.16). Conidia from the wild TSM isolate of *T. viride* did not developed with doses higher than 1.0 $\mu g.mL^{-1}$. The presence of eight mutant colonies of TSM resistant to 5.0 $\mu g.mL^{-1}$ of benomyl fungicide was observed. The resistant mutants showed morphological alterations (mycelium with whitish color and some greenish sectors) observed after five weeks of growth at 25° C with no light, contrasting with a greenish color of the wild TSM isolate.

Table 3. Number of surviving colonies of TSM isolate of *Trichoderma viride* exposed to ultraviolet light and microwave radiation (dose close to 5% survival) and at different concentrations of benomyl fungicide. (mean of five repetitions).

Fungicide concentration (μg.mL-1)	Number of surviving colonies of TSM isolate exposed to ultraviolet light	Number of surviving colonies of TSM isolate exposed to microwave radiation
Control (1)	1004.00 a	-
Control(2)	-	2743.00 a
0.0	46.16 b	189.40 b
1.0	15.83 c	139.00 b
5.0	8.00 c	0.00 c
10.0	0.00 d	0.00 c

Means followed by the same letter do not present any difference among themselves (Tukey test 5%).
Control (1) – treatment with suspension of conidia of TSM not exposed to ultraviolet light and planted in medium without fungicide.
Control (2) – treatment with suspension of conidia of TSM not exposed to microwave radiation and planted in medium without fungicide.

In the experiment with microwave radiation (Table 3), it was used the same ultraviolet criteria for the exposure time for the obtainment of the mutants was used (exposure time close to the 5% survival dose), which equals eight. For the TSM isolate, there were no surviving mutant colonies observed at concentration 5.0 $\mu g.mL^{-1}$ concentration, which did not interfere in the number if colonies developing in treatments with 0.5 and 1.0 $\mu g.mL^{-1}$ doses.

After three successive transfers to PDA medium without benomyl

fungicide, the eight mutants TSM mutants (Table 3) maintained resistance as well as antagonism *in vitro* and did not show differences from the wild TSM isolate in relation to the phytopathogens *Sclerotinia scletotiorum*, *Fusarium* spp. and *Rhizoctonia solani*. The same grades were observed for the antagonism *in vitro* for the wild TSM isolate and for the eight mutants considering the evaluation criteria, where the grade 2.0 (antagonist grows 2/3 over the dish) for the *Fusarium* spp. and *Rhizoctonia solani* and grade 2.5 (antagonist grows from 2/3 to ½ of the dish) for *Sclerotinia sclerotiorum* were found.

The increased exposure time to ultraviolet radiation caused a decreased number of surviving conidia of TSM isolate of *T. viride*. Similar results were obtained by SILVA (1991) and DONZELI *et al*. (1997), such results showed that the decrease of the survival of the primary conidia was proportional to the increase of the radiation doses. STEVENSON & WEIMER (2002) observed a decrease up to 10^3 times in the viability of conidia in suspension irradiated by 10 minutes, and five centimeters away from the ultraviolet radiation source.

These results were confirmed by AZEVEDO (1998), who stated that ultraviolet radiation inhibits microorganism growth. The radiation interrupts the chain of biosynthetic reactions in a determined point, not having forming the final product (VILELLA, 1972).

The decrease in surviving colonies following the increase un exposure time may be caused by the increased temperature. Similar results were obtained by WELT *et al*. (1994), who justified the lethality of *Clostridium* spores by heating and not by the effects of the microwave radiation itself.

Trichoderma viride has a variable behavior in relation to fungicides. It is tolerant to captan, iprodione, etridiazole, PCNB and thiram; however, it is not tolerant to benomyl (PAPAVIZAS, 1980; ABD-EL MOITY *et al*., 1982; SILVA *et al*., 1999). Mutants resistant to 5 μg.mL^{-1} benomyl fungicide were obtained through the exposure of TSM isolate of *Trichoderma viride* to ultraviolet radiation. *Trichoderma* sp. May become resistant to benomyl by the structural alteration of b-tubulin, component of the heterodimers tubulin of the microtubes. It is well known that the fungicides from the benzimidazole group such as benomyl act directly over the microtubes (GOLDMAN *et al.*, 1993). The obtainment of mutants resistant to benomyl was observed by SILVA (1991) through ultraviolet exposure at concentrations up to 1000 μg.mL^{-1}. The action of the radiation is not the same in all spores, which suggests that the organisms present different repair mechanisms, producing surviving colonies resistant to ultraviolet action. Exposure to ultraviolet radiation may be also useful in order to obtain other kinds of mutants. STEVENSON & WEIMER (2002) obtained

three auxotrophic mutants and two morphological mutants with yellow pigmentation of *Trichoderma viride* through the exposure to ultraviolet radiation in order to demonstrate the parasexuality in *Trichoderma*.

The relative ease that the mutants were obtained in the paper through ultraviolet irradiation of conidia suggests that the conidia were predominant uninucleates as observed for other isolates (TOYAMA *et al*., 1984; STASZ *et al*., 1989; SILVA, 1991).

Mutants resistant to benomyl were not obtained with microwave radiation and one of the factors which favor this result may have been the exposure time. The different kinds of radiation produce different biological effects, depending on the waveform, its energy and exposure time (AZEVEDO, 1998). As we worked with two kinds of radiation, microwave and ultraviolet, the most suitable dose for microwave radiation to obtain mutants of *Trichoderma viride* may be different from that which obtains mutants with ultraviolet radiation, 5% survival.

The microwave radiation may have low capacity of inducing mutations at the frequency of 2450MHz, which was used in this paper, due to the fact that it may have affected just the water and not the conidia DNA. According to FUJIKAMA *et al*. (1992), microwave is incapable of breaking the covalent links of the DNA molecule. However, KAKITA *et al*. (1995) showed that the effects of microwave radiation at 2450MHz is distinguishable from the external heat by the fact of being capable of extensively fragmenting the viral DNA (bacteriophages PL-1), what is not observed when used just for the heating of phages at the same temperature.

Conclusion

It was concluded that although both ultraviolet and microwave radiation caused reduction in the survival of conidia in suspension, only ultraviolet radiation was efficient in obtaining stable mutants of TSM isolate of *Trichoderma viride* resistant to 5.0 $\mu g.mL^{-1}$ of benomyl fungicide at exposure time which allows 5% survival in the presented conditions.

References

ABD-EL MOITY T.H.*et al*. Induction of new isolates of *Trichoderma harzianum* tolerant to fungicides and their experimental use for control of white rot of onion. **Phytopathology**, St. Paul ,v.72, p.394-400, 1982.

AZEVEDO, J.L. **Genética de microorganismos**. Goiânia: UFG, 1998. 490p.

BELL D.K. *et al*. *In vitro* antagonism of *Trichoderma* species against fungal plant pathogens. **Phytopathology,** St. Paul**,** v.72, p.379-382, 1982.

CASSIOLATO, A.M.R. **Parasitismo de *Sclerotinia sclerotiorum* (Lib.) de Bary por mutantes de *Trichoderma harzianum***. 1995. 145f. Tese (Doutorado em Agronomia) – Curso de Pós Graduação em Agronomia, USP/ESALQ.

CASSIOLATO, A.M.R.; MELO, I.S. Filtration enrichment method for isolation of auxotrophic mutants of *Trichoderma harzianum* Rifai. **Revista de Microbiologia,** São Paulo, v.30, p.43-46, 1999.

DONZELI V.P. *et al.* Resistência de conídios do fungo *Neozygites* à luz ultravioleta, Salvador, BA, 1997. In: CONGRESSO BRASILEIRO DE ENTOMOLOGIA, 16., 1997, Salvador, BA. **Anais**... Salvador: Sociedade Entomológica do Brasil/EMBRAPA-CNPMF, 1997, 400p., p.147.

EDINGTON L.V.; BARRON, G.L. Fungitoxic spectrum of benzimidazole compounds. **Phytopathology,** St. Paul, v.6, p. 42-44, 1971.

ETHUR L.Z. *et al*. Fungos antagonistas a *Sclerotinia sclerotiorum* em pepineiro cultivado em estufa. **Fitopatologia Brasileira,** Brasília, v.30, p.127-133, 2005.

ETHUR, L.Z. *et al*. Seleção de *Trichoderma* spp. visando ao controle de *Sclerotinia sclerotiorium in vitro*. **Ciência Rural,** Santa Maria, v.31, p.885-887, 2001.

FERNANDES, M,R. **Manual para laboratório de fitopatologia**. Passo Fundo: EMBRAPA/CNPT, 1993. 128 p.

FUJIKAMA, H. *et al*. Kinetics of *Escherichia coli* destruction by microwave irradiation. **Applied and Environmental Microbiology**, USA: ESNP, v.58, p. 920-924, 1992.

GOLDMAN, G.H. *et al*. A point mutation in one of ?-tubulin genes of *Trichoderma viride* confers resistance to the antimitotic drug methyl benzimidazole-2-il-carbamate. **Molecular and General Genetics**, New York, v.240, p.64-68, 1993.

KAKITA, Y. *et al*. Inactivation of lactobacillus bacteriophage PL-1 microwave irradiation. **Microbiological Immunology,** Canada, v.39, p. 571-576, 1995.

MELO, I.S. Agentes microbianos de controle de fungos fitopatogênicos. In: MELO, I.S.; AZEVEDO, J.L. (Ed.). **Controle biológico**. Jaguariúna: EMBRAPA/CNPDA, 1998. cap.1, p.17-68.

MENTEN, J.O.M. *et al*. Efeito de alguns fungicidas no crescimento micelial de *Macrophomina phaseolina* (Tass.) Goid. *in vitro*. **Fitopatologia Brasileira**, Brasília, v.1, p.57-66, 1976.

NEDER, R.N. **Microbiologia: manual de laboratório.** São Paulo: Nobel, 1992. 137p.

PACCOLA-MEIRELLES, L.D. Genética e melhoramento de fungos agentes de controle biológico. In: MELO, I.S.; AZEVEDO, J.L. (Ed.). **Controle biológico**. Jaguariúna: EMBRAPA/CNPDA, 1998. cap. 6, p.171-200.

PAPAVIZAS, G.C. Induced tolerance of *Trichoderma harzianum* to fungicides. **Phytopathology,** St. Paul, v.70, p.691-692, 1980.

SILVA, A.C.F. **Obtenção e caracterização de novos biótipos de *Trichoderma harzianum* Rifai, resistentes a benzimidazóis, através da luz ultravioleta**. 1991. 133f. Dissertação (Mestrado em Agronomia) Curso de Pós Graduação em Agronomia, USP/ESALQ.

SILVA, A.C.F. **Uso da radiação gama para obtenção de mutantes de *Trichoderma harzianum* Rifai e *Trichoderma viride* Pers.: Fr. com capacidade melhorada no controle ao *Sclerotinia sclerotiorum* (Lib.) de Bary**. 1997. 143f. Tese (Doutorado em Agronomia)- Curso de Pós Graduação em Agronomia, USP/ESALQ.

SILVA, A.C.F.; ROSA, C.R.E; MELO, I.S. Sensibilidade de isolados de *Trichoderma* spp. a benomil e iprodione. **Ciência Rural**, Santa Maria, v.29, p.395-399, 1999.

STASZ, T.E. *et al*. Limited vegetative compatibility following intra- and interspecific protoplast fusion in *Trichoderm*a. **Experimental Mycology**, v.13, p. 364–371, 1989.

STEVENSON, D.M.; WEIMER, P.J. Isolation and characterization of a *Trichoderma* strain capable of fermenting cellulose to ethanol. **Applied and Microbiology Biotechnology**, Springer-Verlag , v.59, p.721–726, 2002.

TOYAMA, H. *et al*. Protoplast fusion of *Trichoderma reese*i, using immature conidia. **Applied Environmental Microbiology**, USA: ESNP, v.47, p.363–368, 1984.

VILELLA, G.G. **Bioquímica dos microrganismos**. Pernambuco: UFP, 1972, 290p.

WELT, B.A. *et al*. Effect of microwave radiation on inactivation of *Clostridium sporogenes* spores. **Applied Environmental Microbiology**, USA: ENSP, v.60, p.482-488, 1994.

Submetido em: 02/04/2009
Aceito em: 29/05/2009