ABRATES

Journal of Seed Science
ISSN 2317-1545
www.abrates.org.br/revista

NOTE

Use of 2,3,5-triphenyl tetrazolium chloride for detection of *Fusarium semitectum* viability in soybean seeds

Journal of Seed Science, v.46, e202446021, 2024

http://dx.doi.org/10.1590/2317-1545v46278215

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ABSTRACT: Colorimetric techniques, such as tetrazolium salts and resazurin, are indicators of viability (respiratory activity) of fungi, bacteria, and seeds widely used in different areas of research. The objective of this study was to develop a protocol with two screening methods, qualitative and quantitative, to detect the viability of *Fusarium semitectum* in soybean seeds using 2,3,5-triphenyl tetrazolium chloride (TZ). The experimental design was completely randomized in a triple factorial arrangement (incubation times, TZ concentrations, and inoculum serial dilutions) with levels of 3, 5, and 5 of each factor, respectively, and 3 replications in both methods. For the qualitative method, sensitivity was more satisfactory at 72 hours, at the minimum TZ concentration of 0.1 mg mL^{-1}, and with the inoculum concentration of 10^{-1}. In the quantitative method (spectrophotometry), it was not possible to quantify the colony forming units (CFU) on plates, with the Optical Density (OD) being interfered with by structure of the mecelium in the microtube. Although it is a reproducible methodology, it is conditioned by fungal morphology. The present study allowed easy visualization of the structures of *F. semitectum* in soybean seeds, based on the TZ. However, it was not possible to quantify the inoculum density, due to low spore production, requiring greater adjustments in the methodology.

Index terms: colorimetric techniques, phytopathogens, TZ, viability.

RESUMO: Técnicas colorimétricas, como sais de tetrazólio e resazurina, são indicadores de viabilidade (atividade respiratória) de fungos, bactérias e sementes, amplamente utilizados em diferentes áreas de pesquisa. O objetivo deste estudo foi desenvolver um protocolo com dois métodos, qualitativo e quantitativo, de triagem para detecção da viabilidade de *Fusarium semitectum* em sementes de soja, utilizando 2,3,5-trifenil cloreto de tetrazólio (TZ). O delineamento experimental utilizado foi inteiramente casualizado (DIC) em arranjo fatorial triplo (tempos de incubação, concentrações de TZ e diluições seriadas de inóculo) com níveis de 3, 5 e 5 de cada fator, respectivamente, com 3 repetições em ambos os métodos. Para o método qualitativo, a sensibilidade foi mais satisfatória no tempo de 72h, na concentração mínima de TZ de 0,1 mg mL^{-1} e com a concentração de inóculo 10^{-1}. No método quantitativo (espectrofotometria) não foi possível quantificar as Unidades Formadoras de Colônias (UFC) em placas, sendo a Densidade Óptica (DO) interferida pela estrutura do micélio no microtubo, embora seja uma metodologia reprodutível está condicionada pela morfologia do fungo. O presente estudo permitiu fácil visualização das estruturas de *F. semitectum* em sementes de soja, a partir do TZ, porém não foi possível quantificar a densidade de inóculo devido a baixa produção de esporos precisando de maiores ajustes na metodologia.

Termos para indexação: Técnicas colorimétricas, fitopatogênicos, TZ, viabilidade.

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Received: 09/04/2023.
Accepted: 06/03/2024.

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INTRODUCTION

One of the main challenges of the seed sector is production of good quality seeds in different tropical and subtropical climate conditions. Among the biotic factors limiting soybean production, diseases caused by seed-borne fungi occur with greater frequency, causing qualitative losses associated with physiological quality and quantitative losses associated with yield (Krzyzanowski et al., 2018; Seixas et al., 2020).

Diagnosis of seed-associated pathogens can be adopted as a preventive strategy in disease management because it prevents the introduction of pathogens into the field. Furthermore, the seed health quality test provides traceability of seed lots both in the pest quarantine system and in the production of certified seeds (Bergamin-Filho and Amorim, 2018).

Among the conventional methods, the blotter test stands out by detecting different genera of seed-associated fungi, and it is the method most used to certify the sanitary quality of seed lots (Brasil, 2009; ISTA, 2023). Yet, although it is an efficient method, it requires specialized knowledge in mycology and highly qualified labor. Moreover, it requires time, an average of seven days, for formation of reproductive structures and subsequent analysis. The sum of these factors ends up limiting the number of samples analyzed per day. Screening methods that detect the viability of fungi associated with seeds may be feasible, so as to reduce the number of samples that will be sent to the blotter test, and thus speed the flow of samples in the laboratory.

Colorimetric techniques allow quick, simple, and simultaneous analysis of a large number of samples. To do so, it is necessary to adjust the inoculum concentration to be detected by the spectrophotometer. Investigations applying colorimetric methods with TZ are widely used as markers of cell viability of fungi, bacteria, and seeds (Meletiadis et al., 2000; 2001; Lall et al., 2013; Stiefel et al., 2013; Xu et al., 2015; Alonso et al., 2017; Cen et al., 2018; Grela et al., 2018; Ishiki et al., 2018; Pereira et al., 2019).

In soybean seeds, TZ is widely used to determine the physiological quality of seed lots. Although it is another area of research, the principle is the same used in seed science and technology, based on a reduction reaction in viable cells, which form a stable, red, and non-diffusible compound called triphenylformazan (França-Neto and Krzyzanowski, 2018; Pereira et al., 2019).

Therefore, the aim of this study was to develop a screening protocol to detect the viability of *F. semitectum* in soybean seeds using TZ.

MATERIAL AND METHODS

The study was developed in the Seed Pathology and Phytopathogenic Fungi Laboratory (LPSFF) of the Plant Health Department of the Faculdade de Agronomia Eliseu Maciel - *Universidade Federal de Pelotas* (FAEM-UFPel), campus of Capão do Leão, RS, Brazil. To conduct the studies, soybean seeds artificially inoculated with *F. semitectum* were used. The seeds were inoculated using the potato sucrose agar (PSA) medium, and the seeds were deposited in a single layer in a culture medium containing the fungus for a period of 48 h in an incubation room, with a photoperiod of 12 h and temperature of 23 ± 2 °C.

Two methods, one qualitative and one quantitative, were tested to detect *F. semitectum* viability in soybean seeds using TZ. Sample incubation times, TZ concentrations, and serial dilutions of the inoculum were considered in both methods.

To extract the fungus from the seeds, a 200-seed subsample was placed in a hermetically sealed, sterile glass container containing sterilized water in a sufficient quantity to cover the seeds with a 2-cm layer of water. The containers were placed under continuous shaking in an incubation room for 8 h at 23 ± 2 °C. After that period, a 20-mL subsample and a 100-mL counter-sample of the inoculum suspension were extracted, discarding the seeds. The time chosen for incubation was determined based on studies performed on *Sclerotinia sclerotiorum* (Grabicoski et al., 2015; Ramiro et al., 2019) and *Phomopsis* spp. (Jaccoud-Filho et al., 1996; Jaccoud-Filho et al., 2002).

In the negative control (C-), the extraction liquid from healthy seeds selected by the blotter test (5 days) was used. A positive control (C+) used the extraction liquid obtained from seeds inoculated with *F. semitectum* seeded in a potato dextrose (PD) mixture and PD with antibiotic mixtures, with concentrations of 0.025, 0.050, and 0.075 mg.mL^{-1} of chloramphenicol, which was selective in controlling the bacteria. The controls were maintained without addition of TZ.

In 2-mL microtubes, containing 1.8 mL of PD, TZ was added at the concentrations of 0.1 mg.mL^{-1}, 0.5 mg.mL^{-1}, 1.0 mg.10 mL^{-1}, 5.0 mg.mL^{-1}, and 10.0 mg.mL^{-1}, followed by addition of a 0.2 mL aliquot of the extraction liquid, separately, at dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}. Incubation temperature and time were determined according to the method described in the Clinical and Laboratory Standards Institute (CLSI) standard document M38-A (Lage et al., 2013; Wayne, 2017); and Carrillo-Muñoz et al. (2004) and Pujol et al. (1997) recommended 35 °C for 46-50 h and 25-35 °C for 48-72 h, respectively. Then, with some modifications for *Fusarium* spp., the microtubes were incubated at 25 °C for 0 h, 48 h, and 72 h, placing them on an orbital shaker in continuous darkness for later evaluation. The microtubes with the treatments were used for both the qualitative and quantitative methods.

A completely randomized experimental design (CRD) was used in a triple factorial arrangement (sample incubation times, TZ concentration, and inoculum concentration) with levels of 3, 5, and 5 of each factor, respectively, with three replications, for both the qualitative method and the quantitative method.

To classify the results of the qualitative method, two diagnostic cases (classes) were predicted in the fungal biomass, determined by the ability of the method to detect change in color to red (colored mycelia) because it indicates the viability of the *F. semitectum* inoculum. In this classification, the dichotomous dependent (response) variable, called colorimetric reaction, represented 1 in the case of a colorimetric reaction and 0 in the case of no colorimetric reaction. The following measures of incubation time, TZ concentrations, and serial dilution of the inoculum were taken as the independent (explanatory) variables.

Based on the microtubes visualized, fungal growth was confirmed through application of the pour-plate seeding technique and incubation with continuous darkness and temperature of 23 ± 2 °C for 5 days. The binary results were 0/1 (-/+), that is, presence/absence of *F semitectum*.

The results obtained from the microtubes (qualitative method) were analyzed using the binomial logistic regression model (logit link function), classification matrix (sensitivity, specificity, accuracy, and precision) and Relative Operating Characteristic *(*ROC) (sensitivity, specificity, and AUC – Area Under the ROC Curve). The chi-square (X^2) was used to evaluate the significance level among the explanatory variables. In the classic standard method (pour plate) (Apha, 1992; Collins and Lyne, 1989), the plates were analyzed with the binomial logistic regression model, and the significance level using the test.

To develop the quantitative method for detecting *F. semitectum* in soybean seeds using the TZ, the optical density (OD) or absorbance (A) and the Colony Forming Units (CFU) were evaluated following the methodology proposed for bacteria and fungi by different authors (Vivas and Torres, 1998; Carrillo-Muñoz et al., 2004; Stiefel et al., 2013) adapted for the agricultural area.

The calibration line was created with the five serial dilutions of the inoculum suspension in PD (liquid medium) and the blank (same composition as the PD, without the inoculum). Each one of these serial dilutions was homogenized for later evaluation by placing each microtube in a vortex shaker for 15 seconds and then leaving it in an orbital shaker for 30 minutes in an incubation room at 23 ± 2 °C under continuous shaking.

The microtubes containing the treatments were indirectly quantified using the spectrophotometry technique, measuring the optical density (OD) or the absorbance (A) in the cuvette of the spectrophotometer (1.5 mL) at 550 nm. The OD value was expressed as the mean of the values observed in each replicate.

To check the CFU, a 100-µL aliquot of inoculum was seeded on an empty Petri dish and then the PDA medium was poured on according to the classic standard method of the pour-plate technique and incubated in the chamber in continuous darkness at 23 ± 2 °C for 3 to 5 days. The CFUs on the plates were analyzed for fungal growth, and the results were expressed in CFU.mL^{-1}.

The dependent (response) variable was constituted by the OD values produced by the fungi of each microdilution, and the independent variable by the CFU.mL^{-1} of the same microdilutions quantified by the classic (plates) method.

In the quantitative method, the data of CFU.mL^{-1} and OD were analyzed using simple linear regression and fitted to a regression equation. Analysis of variance (ANOVA) of the model, considering the mean values, was carried out using Fisher's test (with a significance level of 5% probability). The model and response variable were estimated using the coefficient of determination (R^2).

RESULTS

In the binary logistic regression model of the qualitative method, the explanatory or independent variable (TZ concentration) has an odds ratio (OR) OR < 1 of having an effect on the red color reaction of the mycelia (Triphenylformazan), indicating negative relationships with positive cases. With an increase in the concentration, there was a reduction in visualization of the fungal biomass, although the results were not significant (*p* value = n.s.).

In contrast, the incubation time and the dilution of the inoculum (explanatory variables) have OR > 1 in relation to the Triphenylformazan, indicating positive relationships with positive cases. It is thus observed that the chances of a positive diagnosis are greater when the time and inoculum increase; otherwise, it occurs with the TZ concentration. The results of the chi-square test (X^2) were highly significant statistically (*p* value = < 0.001) for both the predictors (time and inoculum).

The results obtained from the classic standard method show that the chances of fungal growth, OR < 1, have negative relationships of positive diagnosis in the TZ variable. That is due to the fact that higher concentrations considerably reduce positive cases. They exhibit statistically significant X^2 results with *p* value = < 0.001. The time variable has OR > 1, which indicates positive relationships of positive diagnosis in the cases, and the results are statistically significant (X^2) with *p* value = < 0.01, for there was fungal growth in nearly all the microtubes that were seeded on plates.

A total of 225 observations were evaluated, resulting in 152 positive cases and 73 non-positive cases. The area under the curve (AUC, discriminating measurement) for the model was AUC = 0.957 (Figure 1), higher than 90% (a nearly perfect predictive ability).

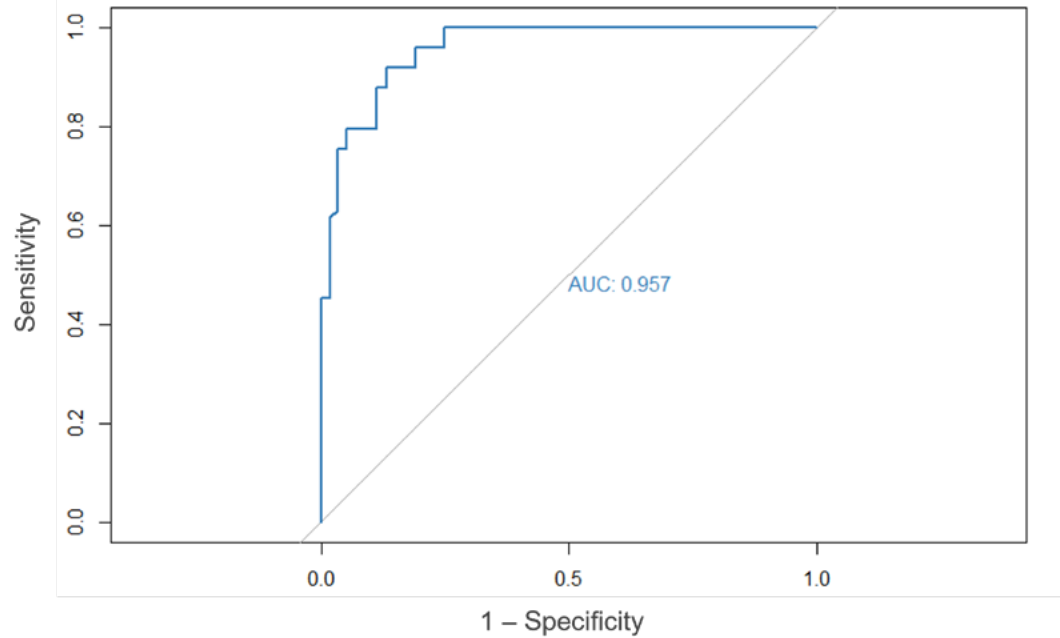

Figure 1. ROC curve analysis of sensitivity and specificity for the model was AUC = 0.957.

The classification matrix discriminates classes into true positive (TP), true negative (TN), false negative (FN), and false positive (FP). Using the matrix, test data were determined according to the incubation times in microtubes (Table 1).

Based on the classification matrix for time 0 h, without incubation, positive cases of TP were not detected (0.0%), and none of the inoculum dilutions exhibited color change, even with TZ (Figure 2), since there was no respiratory activity of *F. semitectum* in this period. The best response was obtained at 72 h of incubation, with 33.0% (TP) of the cases showing a reduction reaction to the TZ (triphenylformazan) (Figure 2), resulting in a sensitivity of 65.0%. The specificity found was 100.0%, for there was no reduction reaction of the TZ (without triphenylformazan) in the proportion of TN (50.0%). A high precision value was calculated, 100.0%, because it classifies 0.0% FP as a predictive value. Thus, the accuracy of the model, a measure that represents the rate (or proportion) of right responses in the total, was 83.0%.

For the TZ concentrations (Table 2), the classification matrix shows that the reduction reaction (colored mycelia) ranges from 0.1 to 1.0 (mg.mL^{-1}) in the TZ concentrations, and was greater at 0.5, with 27.0% (TP). When the concentration increases to 5.0 mg mL^{-1} of TZ, there is a tendency of an inverse TP relationship. Consequently, the greatest percentage of sensitivity was 62.0% in relation to the proportion of TP correctly predicted at the 0.5 mg.mL^{-1} concentration; and the metric that represents accuracy was greater at this concentration, at 77.0%. The highest specificity of the TN cases was 96%, and its precision was higher than the other concentrations (1.0), at 90%.

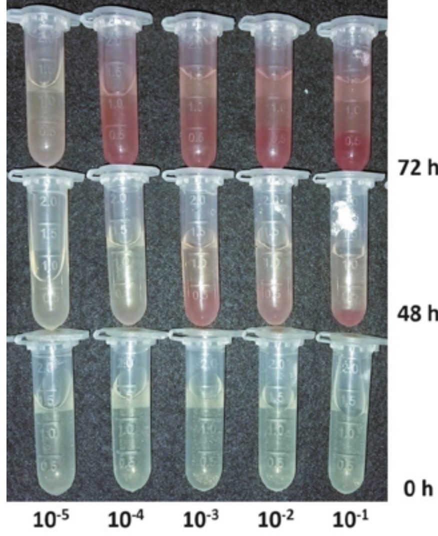

Figure 2. Microtubes in serial dilutions of inoculums and incubation times analyzed at the concentration of 0.5 mg.mL^{-1}.

Table 1. Classification matrix that detects the reaction of 2,3,5-triphenyl tetrazolium chloride (TZ) in *Fusarium semitectum* as a function of incubation times expressed as a percentage.

Colorimetric reaction in microtubes						
Triphenylformazan	Time (h)					
	0 h		48 h		72 h	
	P	A	P	A	P	A
Positive	0.0^{TP}	0.0^{FP}	16.0^{TP}	11.0^{FP}	33.0^{TP}	0.0^{FP}
Negative	50.0^{FN}	50.0^{TN}	23.0^{FN}	50.0^{TN}	17.0^{FN}	50.0^{TN}
Totals	50.0	50.0	39.0	61.0	50.0	53.0

P: Presence of the fungus, A: Absence of the fungus.

Table 2. Classification matrix that detects the reaction of 2,3,5-triphenyl tetrazolium chloride (TZ) in *Fusarium semitectum* as a function of salt concentrations expressed as a percentage.

Triphenylformazan	Colorimetric reaction in microtubes – Doses (mg.mL^{-1}) 0.1 P	0.1 A	0.5 P	0.5 A	1.0 P	1.0 A	5.0 P	5.0 A	10.0 P	10.0 A
Positive	23.0^{TP}	10.0^{FP}	27.0^{TP}	7.0^{FP}	21.0^{TP}	2.0^{FP}	3.0^{TP}	0.0^{FP}	3.0^{TP}	0.0^{FP}
Negative	17.0^{FN}	50.0^{TN}	17.0^{FN}	50.0^{TN}	27.0^{FN}	50.0^{TN}	47.0^{FN}	50.0^{TN}	47.0^{FN}	50.0^{TN}
Totals	40.0	60.0	43.0	57.0	48.0	52.0	50.0	50.0	50.0	50.0

P: Presence of the fungus, A: Absence of the fungus.

Table 3. Classification matrix that detects the reaction of 2,3,5-triphenyl tetrazolium chloride (TZ) in *Fusarium semitectum* as a function of the inoculum potentials expressed as a percentage.

Triphenylformazan	Colorimetric reaction in microtubes – Serial microdilutions (Inoculum) 10^{-5} P	10^{-5} A	10^{-4} P	10^{-4} A	10^{-3} P	10^{-3} A	10^{-2} P	10^{-2} A	10^{-1} P	10^{-1} A
Positive	8.0^{TP}	7.0^{FP}	17.0^{TP}	2.0^{FP}	13.0^{TP}	3.0^{FP}	17.0^{TP}	3.0^{FP}	23.0^{TP}	3.0^{FP}
Negative	36.0^{FN}	50.0^{TN}	31.0^{FN}	50.0^{TN}	33.0^{FN}	50.0^{TN}	30.0^{FN}	50.0^{TN}	23.0^{FN}	50.0^{TN}
Totals	43.0	57.0	48.0	52.0	47.0	53.0	47.0	53.0	47.0	53.0

P: Presence of the fungus, A: Absence of the fungus.

For the inoculum dilutions, the classification matrix shows that at high dilutions, sensitivity declines, and the TP are lower than the FN, except at the lowest inoculum dilution (10^{-1}) at 50.0%. The specificity or true negative (TN) rate had a value of 94.0%, accuracy of 73.0%, and precision of 88.0% in this inoculum dilution (Table 3).

Considering the test conditions, the sensitivity of the method was affected by detection of the change of color to red (colored mycelia) in each microtube, and the most satisfactory time was 72 h at the TZ concentration of 0.5 mg.mL^{-1} and at the inoculum dilution of 10^{-1} (Figure 2).

In the quantitative method, determined by the classic standard method and spectrophotometry, CFU.mL^{-1} and OD, respectively, could not be statistically analyzed. One of the limitations was the formation of colonies for counting on plates, and the production of conidia was insufficient in seed inoculation.

DISCUSSION

In this study, a qualitative screening method was developed as a rapid protocol to detect the viability of the fungus *F. semitectum*, through TZ, using a suspension from a sample extracted from seeds. The TZ-test is characterized as an indirect, efficient, and low-cost method for detection of the fungus based on reduction of the tetrazolium salt to triphenylformazan as a reliable measure for visual inspection by change in color.

In clinical mycology using tetrazolium colorimetry, a study carried out by Levitz and Diamond (1985) found a linear relationship between the inoculum and reduction of 3-(4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl-2H-tetrazolium bromide (MTT) of filamentous fungi, showing > 99.0% viability of red-colored hyphae of *Aspergillus fumigatus* and *Rhizopus oryzae* compared to 0.0% dead hyphae. That explains that as the fungal biomass (colored mycelia) increases,

the MTT and the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) are also reduced in the viable hyphae (Levitz and Diamond, 1985; Freimoser et al., 1999; Lewis et al., 2005).

The study showed that MTT at concentrations of 0.125 mg.mL^{-1} and below had no effect on fungal growth, whereas high concentrations (> 0.125 mg.mL^{-1}) of salt are toxic to *Scedosporium* spp. and *Fusarium* spp., showing levels of cytotoxicity in the fungal biomass (Meletiadis et al., 2000). Furthermore, time does not affect survival, but the application of excessive doses over time favors the effect of the tetrazolium reaction, causing slowed growth or physiological death.

Research carried out by Vallejo et al. (2010) compared two types of salts, 2-(4-iodophenyl)-3-(4-dinitrophenyl)-5-phenyl-tetrazolium chloride (INT) and XTT in bacteria. The INT was insoluble and difficult to read, whereas the XTT had rapid reduction (12 h) and solubility of the salt. Furthermore, the salt reduction is sensitive to factors such as temperature, incubation time, pH of the medium, type of microorganism, and salt concentration.

Cytotoxicity in some bacteria to tetrazolium salts, such as INT; XTT; and 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) at the recommended concentration, shows a negative effect on microbial density (Bensaid et al., 2000; Hatzinger et al., 2003; Mccluskey et al., 2005).

Lage et al. (2013) report that the *Fusarium* genus has a filamentous nature and limits the formation of colonies to quantify the fungal biomass. Furthermore, the results of the quantitative method do not follow the CFU acceptance criteria described in the classic counting method by international protocols, which requires around 30-300 (Apha, 1992) and 25-250 (Collins and Lyne, 1989) colonies per plate.

In this study, the TZ is presented as an indirect method to detect the viability of fungi associated with seeds, and it can be extrapolated to other crops and pathogens. Tetrazolium is a reagent that has been extensively studied and used in research. Our approach increases the impact on the development of colorimetric techniques for detecting viability by spectrophotometry.

CONCLUSIONS

The detection method using 2,3,5-triphenyl tetrazolium chloride (TZ) as a screening test to detect the viability of *F. semitectum* in seeds is viable under the conditions in which the study was conducted.

The time, the TZ concentration, and the inoculum dilution directly affect the result. The most satisfactory conditions were time of 72 h, a TZ concentration of 0.5 mg.mL^{-1}, and inoculum potential of 10^{-1}.

In the quantitative method, it was not possible to obtain satisfactory results. It is necessary to adjust the CFU.mL^{-1} and OD of the fungus.

ACKNOWLEDGMENTS

The authors thank the *Universidade Federal de Pelotas, Programa de Pós-Graduação em Fitossanidade*; the Organization of American States (OAS); the *Grupo Coimbra de Universidades Brasileiras*; and the *Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil* (CAPES) for granting a scholarship, as well as the Facultad de Ingeniería Agronómica - Universidad Nacional del Este (FIA-UNE) for their continued support in my professional training.

REFERENCES

ALONSO, B.; CRUCES, R.; PÉREZ, A.; SÁNCHEZ-CARRILLO, C.; GUEMBE, M. Comparison of the XTT and resazurin assays for quantification of the metabolic activity of Staphylococcus aureus biofilm. *Journal of Microbiological Methods*, v.139, p.135–137, 2017. https://doi.org/10.1016/j.mimet.2017.06.004

APHA. COMPENDIUM of methods for the microbiological examination of foods. 3 ed. ed. Washington (USA): *American Public Health Association*, 1992. https://doi.org/10.2105/MBEF.0222

BENSAID, A.; THIERIE, J.; PENNINCKX, M. The use of the tetrazolium salt XTT for the estimation of biological activity of activated sludge cultivated under steady-state and transient regimes. *Journal of Microbiological Methods*, v.40, n.3, p.255–263, 2000. https://doi.org/10.1016/s0167-7012(00)00130-5

BERGAMIM-FILHO, A.; AMORIM, L. Parte III Controle de doenças: 14. Princípios gerais de controle. In: AMORIM, L.; REZENDE, J. A. M.; BERGAMIN FILHO, A. (Eds.). *Manual de Fitopatologia*. 5. ed. ed. Ouro Fino: *Agronômica CERES*, 2018. p.215–227.

BRASIL. Ministério da Agricultura, Pecuária e Abastecimento. *Manual de Análise Sanitária de Sementes*. Brasília: Mapa/ACS, 2009, 200p. https://www.gov.br/agricultura/pt-br/assuntos/insumos-agropecuarios/insumos-agricolas/sementes-e-mudas/publicacoes-sementes-e-mudas/manual-de-analise-sanitaria-de-sementes

CARRILLO-MUÑOZ, A.J.; QUINDÓS, G.; DEL VALLE, J.M.; HERNÁNDEZ-MOLINA, J.M.; SANTOS, P. Antifungal activity of amphotericin B and itraconazole against filamentous fungi: Comparison of the sensititre yeast one® and NCCLS M38-A reference methods. *Journal of Chemotherapy*, v.16, n.5, p.468-473, 2004. https://doi.org/10.1179/joc.2004.16.5.468

CEN, Y.K.; LIN, J.G.; WANG, J.Y.; LIU, Z.Q.; ZHENG, Y.G. Colorimetric assay for active biomass quantification of Fusarium fujikuroi. *Journal of Microbiological Methods*, v.155, p.37–41, 2018. https://doi.org/10.1016/j.mimet.2018.11.009

COLLINS, C.H.; LYNE, P.M. *Metodos microbiologicos*. 5. ed. Zaragosa: ACRIBIA, 1989.

FRANÇA-NETO, J.B.; KRZYZANOWSKI, F.C. *Metodologia do teste de tetrazólio em sementes de soja Metodologia do teste de tetrazólio em sementes de soja*. Londrina, PR: Embrapa, 2018. https://www.embrapa.br/busca-de-publicacoes/-/publicacao/1098452/metodologia-do-teste-de-tetrazolio-em-sementes-de-soja

FREIMOSER, F.M.; JAKOB, C.A.; AEBI, M.; TUOR, U. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a fast and reliable method for colorimetric determination of fungal cell densities. *Applied and Environmental Microbiology*, v.65, n.8, 1999. https://doi.org/10.1128/aem.65.8.3727-3729.1999

GRABICOSKI, E.M.G.; FILHO, D.S.J.; PILEGGI, M.; HENNEBERG, L.; PIERRE, M.L.C.; VRISMAN, M.C.; DABUL, A.N.G. Rapid PCR-based assay for Sclerotinia sclerotiorum detection on soybean seeds. *Scientia Agricola*, v.72, n.1, p.69–74, 2015. http://dx.doi.org/10.1590/0103-9016-2013-0395

GRELA, E.; KOZŁOWSKA, J.; GRABOWIECKA, A. Current methodology of MTT assay in bacteria – A review. *Acta Histochemica*, v.120, n.4, p.303-311, 2018. https://doi.org/10.1016/j.acthis.2018.03.007

HATZINGER, P.B.; PALMER, P.; SMITH, R.L.; PEÑARRIETA, C.T.; YOSHINARI, T. Applicability of tetrazolium salts for the measurement of respiratory activity and viability of groundwater bacteria. *Journal of Microbiological Methods*, v.52, n.1, p.47–58, 2003. https://doi.org/10.1016/S0167-7012(02)00132-X

ISHIKI, K.; NGUYEN, Q. D; MORISHITA, A; NAGAOKA, T. Electrochemical Detection of Viable Bacterial Cells Using a Tetrazolium Salt. *Analytical Chemistry*, v.90, n.18, 2018. https://doi.org/10.1021/acs.analchem.8b02404

ISTA. International Seed Testing Association. *Seed health testing*. In: International Rules for Seed Testing. Int. Seed Test. Assoc. Zürichstr: ISTA, 2023. p.7-1-7-6.

JACCOUD-FILHO, D.S.; MATIELLO, R.R.; TAYLOR, E.; BATES, J.; LEE, D.; MORAIS, M.H. Diagnose molecular de fungos em sementes. *Revisão Anual de Fitopatologia de Plantas*, v.10, p.287–331, 2002.

JACCOUD-FILHO, D.S.; LEE, D.; REEVES, J.C.; YORINORI, J.T. A PCR-basedd test for detection of Phomopsis phaseoli f. sp. Meridionalis from soya bean seeds. In: HUTCHINS, J.D; REEVES, J.C. (Org.). 2nd ISTA - PDC Symp. SEED Heal. Test. Towar. 21ST CENTURY. Cambridge: CAB International, 1996. p. 35.

KRZYZANOWSKI, F.C.; FRANÇA-NETO, J.D.B.; HENNING, A.A. *A alta qualidade da semente de soja: fator importante para a produção da cultura*. Circular técnica, v.136, n.1, p.1-24, 2018. https://ainfo.cnptia.embrapa.br/digital/bitstream/item/177391/1/CT136-online.pdf

LAGE, L.; PANIZO, M.M.; FERRARA, G.; REVIAKINA, V. Validación del inóculo por densitometría para las pruebas de susceptibilidad a los antifúngicos en especies del género Fusarium. *Revista de la Sociedad Venezolana de Microbiología*, v.33, n.1, p.46–52, 2013. https://ve.scielo.org/scielo.php?script=sci_arttext&pid=S1315-25562013000100010

LALL, N.; HENLEY-SMITH, C.J.; CANHA, M.N.; OOSTHUIZEN, C.B.; BERRINGTON, D. Viability Reagent, PrestoBlue, in Comparison with Other Available Reagents, Utilized in Cytotoxicity and Antimicrobial Assays. *International Journal of Microbiology*, v.2013, n.1, 2013. https://doi.org/10.1155/2013/420601

LEVITZ, S.M.; DIAMOND, R.D. A rapid colorimetric assay of fungal viability with the tetrazolium salt MTT. *Journal of Infectious Diseases*, v.152, n.5, 1985. https://doi.org/10.1093/infdis/152.5.938

LEWIS, R.E.; WIEDERHOLD, N.P.; KLEPSER, M.E. In vitro pharmacodynamics of amphotericin B, itraconazole, and voriconazole against Aspergillus, Fusarium, and Scedosporium spp. *Antimicrobial Agents and Chemotherapy*, v.49, n.3, p.945–951, 2005. https://doi.org/10.1128/aac.49.3.945-951.2005

MCCLUSKEY, C.; QUINN, J.P.; MCGRATH, J.W. An evaluation of three new-generation tetrazolium salts for the measurement of respiratory activity in activated sludge microorganisms. *Microbial Ecology*, v.49, n.3, 2005. https://doi.org/10.1007/s00248-004-0012-z

MELETIADIS, J.; MEIS, J.F.; MOUTON, J.W.; DONNELLY, J.P.; VERWEIJ; P.E. Comparison of NCCLS and 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) methods of in vitro susceptibility testing of filamentous fungi and development of a new simplified method. *Journal of Clinical Microbiology*, v.38, n.8, p.2949–2954, 2000. https://doi.org/10.1128/jcm.38.8.2949-2954.2000

MELETIADIS, J.; MOUTON, J.W.; MEIS, J.F.; BOUMAN, B.A.; DONNELLY, P.J.; VERWEIJ, P.E.; EUROFUNG, R.†. Comparison of spectrophotometric and visual readings of NCCLS method and evaluation of a colorimetric method based on reduction of a soluble tetrazolium salt, 2,3-bis {2-methoxy-4-nitro-5-[(sulfenylamino) carbonyl]-2H- tetrazolium-hydroxide}, for antifung. *Journal of Clinical Microbiology*, v.39, n.12, p.4256–4263, 2001. https://doi.org/10.1128%2FJCM.39.12.4256-4263.2001

PEREIRA, D.F.; BUGATTI; P.H. LOPES, F.M.; SOUZA, A.L.; SAITO, P.T. Contributing to agriculture by using soybean seed data from the tetrazolium test. *Data in Brief*, v.23, p.103652, 2019. http://dx.doi.org/10.1016/j.dib.2018.12.090

PUJOL, I.; GUARRO, J.; SALA, J.; RIBA, M.D. Effects of incubation temperature, inoculum size, and time of reading on broth microdilution susceptibility test results for amphotericin B against *Fusarium*. *Antimicrobial Agents and Chemotherapy*, v.41, n.4, 1997. https://doi.org/10.1128/aac.41.4.808

RAMIRO, J.; CIAMPI-GUILLARDI, M.; CALDAS, D. G. G.; MORAES, M. H. D.; BARBIERI, M. C. G.; PEREIRA, W. V.; MASSOLA, N. S. Quick and accurate detection of Sclerotinia sclerotiorum and Phomopsis spp. in soybean seeds using qPCR and seed-soaking method. *Journal of Phytopathology*, v.167, n.5, p.273–282, 2019. https://doi.org/10.1111/jph.12796

SEIXAS, C.D.S.; NEUMAIER, N.; Balbinot Junior, A.A; Krzyzanowski, F.C.; Leite, R.M.V.B. de C. *Tecnologias de Produção de Soja*. 1ª edição ed. Paraná: Londrina: Embrapa Soja, 2020. https://ainfo.cnptia.embrapa.br/digital/bitstream/item/223209/1/SP-17-2020-online-1.pdf

STIEFEL, S.; GUMIY, D.; CANALIS, M.; SIROSKI, P.; PICCO, E.; ORTEGA, H.; FORMENTINI, E. Desarrollo de un método colorimétrico en microplaca para determinar la cinética de crecimiento de Staphylococcus aureus. *FAVE Sección Ciencias Veterinarias*, v.12, n.1–2, p.131–42, 2013. https://doi.org/10.14409/favecv.v12i1/2.4551

VALLEJO, E.; YANINE, H.; AUGUSTO, F. Aplicación de sales de tetrazolio de nueva generación (Xtt) para la estimación del número más probable. *Acta Biológica Colombiana*, v.15, n.3, p.75–90, 2010. https://www.redalyc.org/pdf/3190/319027886006.pdf

VIVAS, J.R.C.; RODRÍGUEZ, J.M.T. Método espectrofotométrico en la preparación del inóculo de hongos dematiáceos. *Revista Iberoamericana de Micología*, v.15, p.155-157, 1998. https://www.reviberoammicol.com/1998-15/155157.pdf

WAYNE, P.A. Reference method for broth dilution antifungal susceptibility testing of yeasts; Approved standard - 3th ed. CLSI standard M38. Clinical and Laboratory Standards Institute (CLSI), v.37, n.15, p.1–49, 2017. https://clsi.org/media/1461/m27a3_sample.pdf.

XU, M.; MCCANNA, D. J.; SIVAK, J. G. Use of the viability reagent PrestoBlue in comparison with alamarBlue and MTT to assess the viability of human corneal epithelial cells. *Journal of Pharmacological and Toxicological Methods*, v.71, p.1–7, 2015. http://dx.doi.org/10.1016/j.vascn.2014.11.003

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