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Incubation period and fungi identification in seeds of *Salvia hispânica L.*

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Abstract

Seed sanitary quality is an essential factor in crop success, and through the evaluation of fungi contamination of seeds, measures can be adopted to control and avoid the transferability of this contamination to the plants. Equally important is the method used to verify the occurrence and identify the species, especially when it comes to crops that have not yet been studied, such as chia, which has a mucilaginous layer that may present some interference in the development of fungi. This study's objective was to identify the genera of fungi present in lots of saved chia seeds, define the best period for their evaluation and its transmissibility to the plantlet. Eight lots from saved seeds of chia were analyzed into two evaluations at seven and 14 days of incubation via the Blotter test method, in which fungi were identified by their reproductive structure using a stereomicroscope. Besides, the transmissibility rate from seed to plantlet of the most incident fungus was evaluated, through the direct isolation method. All estimated seed lots of chia showed fungi contamination, with emphasis on Fusarium sp., Phomopsis sp., Penicillium sp., and Aspergillus sp. The best evaluation period to Phomopsis sp. its at 14 days of incubation by the Blotter test method. The genus Fusarium sp. presents a high transmissibility rate from seed to plantlet.

Keywords: Blotter test, chia, fungi

Introduction

Chia (*Salvia hispânica* L.) is a herbaceous plant, produces seeds that are used as food due to its nutritional properties (Ayerza & Coates, 2011), and it is a source of proteins and polyunsaturated fatty acids (Coelho & Salas-Mellado, 2014). Because it is propagated via seeds, the sanitary quality is a factor of significant relevance in its crop production because of the possibility of the presence of pathogens associated with the seeds, mainly fungi (Barrocas & Machado, 2010). The presence of these microorganisms is due to several factors in the production field, and in the operations of harvest and post-harvest of seeds, affecting the quality of the lot and decreasing its germination capability (Schuch et al., 2013). Thus, it is crucial to evaluate possible differences among seed lots because they can go through different processes during its production that affects not only its physiological but also its sanitary quality.

Fungi as *Fusarium* sp., *Aspergillus* sp., and *Penicillium* sp. are among the most common found on seeds being responsible for its deterioration before harvest and during the storage period. Besides, those pathogens have the potential to produce mycotoxin that can cause food poisoning through the ingestion of contaminated food (Kempken & Rohlfs, 2010; Rosseto et al., 2015). Certified seeds are produced under rigorous patterns to reach the attributes of physiologic and sanitary qualities. These seeds have proof of origin, varietal purity high physiologic quality, and are free of pathogens (Bellé et al., 2016). However, many growers use their seeds, called saved seeds, which can fail to present the desired characteristics because they are not produced in the required patterns. Saved seeds are one of the main factors responsible for the dissemination of pathogens in the crop environment (Tozzo & Peske, 2008).

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on seeds, detect their occurrence, and transmissibility is important to decide the best measure to control disease (Herrera-Chan et al., 2019). In the sanitary analysis of the seeds manual (Brasil, 2009), there are methodologies for the determination of pathogen presence. Even that the Blotter test for 7-8 days of incubation is one of the most widely-used to the crops of agronomic interest, it is crucial to evaluate modifications or adaptations for species that are not broadly studied, even more for chia because it has a mucilaginous layer that can interfere in fungal development. Besides, it is always possible to adjust methodologies or include new technologies for evaluation, giving continuity to research (Marcos Filho et al., 2009), and improving the knowledge over different species.

This study's objective was to identify the different genera of fungi present in lots of saved chia seeds, define the best evaluation period, and the transmissibility from seeds to plantlets.

Material and Methods

The study was divided into two experiments conducted at the Seed Analysis Laboratory at the Universidade Tecnológica Federal do Paraná (UTFPR), Câmpus Dois Vizinhos. Eight lots of saved chia seeds were evaluated (identified as lots 1 through 8), produced by different growers, during the safrinha period from April to July in the west region of Parana state, Brazil, and the cultivar identified as "common". When seeds arrived at the laboratory, samples from the lots were placed in paper envelopes and stored in a cold and dry (10 °C and 20% of relative humidity) for four months before the start of the experiments.

The first trial was conducted to identify and quantify the fungi genera, using a completely randomized design, in a 8 x 2 factorial scheme, being eight lots and two evaluation times, with four repetitions of 100 seeds from each lot. The methodology used for seed incubation in a filter paper substrate is described in the Manual of Sanitary Analysis of Seed (Brasil, 2009), also called the Blotter Test. Seeds were placed in transparent polypropylene boxes from the "gerbox" type, with dimensions of 11x11x3 cm, on two blotting paper sheets, dampened with 2.5 times their mass with distilled water. To avoid seed germination, in the imbibition, water was added 5ppm pf the 2,4-D in the concentration of 806.0 g L^{-1}. The blotting paper was autoclaved for 30 minutes, and all used material went through asepsis with alcohol 70%. After the seeds were placed on the substrate, the gerbox® boxes were sealed with tape and were incubated in a BOD chamber, at the temperature of 25 ±1 °C, with a 12-hour photoperiod.

At seven and 14 days after incubation, the evaluations for fungi identification were made, and the best period to the evaluation was also assessed. During the visual evaluations with a stereoscope with 80x magnification, the existence of fungi on the seed was noted according to the genus, identified by their reproductive structures. Results were expressed as a percentage.

The second trial evaluated the transmission rate from seed to plantlet of the fungus that was mostly present in the samples from the evaluated lots (*Fusarium* sp.). The fungus isolation was made with the "direct isolation" method. The conidia and mycelium isolates were taken from evaluated seeds in the first trial and grew in a Petri dish with a PDA (potato dextrose agar) media. After five days of growth, when the fungus achieved all the radius space of the dish, 200 seeds of lot 3, which had the highest incidence of *Fusarium* sp., were placed in the dish, having direct contact with the fungus and were under inoculation for 72 hours.

After this period, seeds were placed in gerbox® boxes on the sterilized blotting paper and dampened as already described, and left under incubation for 15 days. Four repetitions of 50 seeds were used. The transmissibility of the fungus from the seed to the plantlets was assessed in the sixteenth day, being considered as infected the seedlings with symptoms and signals of the fungus. In the evaluation of the transmissibility rate, the formula adapted from Teixeira & Machado (2003), in which:

$$IR: PIR\ x\frac{100}{SIR}$$

TT=infection rate;
PIR = plantlet infection rate;
SIR= seed infection rate;

After compilation, the data from the first trial was submitted to the normality test and the analysis of variance (ANOVA) by the F test and means compared by the Tukey test at 5 % probability level.

Results and Discussion

All lots evaluated in this study presented fungi incidence, being detected sixteen genera: *Fusarium* sp., *Penicillium* sp., *Aspergillus* sp., *Phomopsis* sp., *Cladosporium* sp., *Nigrospora* sp., *Rhizoctonia* sp., *Alternaria sp.*, *Chaetomium* sp., *Epicoccum* sp., *Rhizopus* sp., *Periconia* sp., *Trichoderma* sp., *Cercospora* sp., *Corinespora* sp. and *Bipolaris* sp.. However, in the exception of the first four lots, the others presented a low percentage of incidence in both evaluations (mean is inferior to 1.5%), with no statistical difference among lots and evaluation periods and are not showed in this study.

Different genera were also found in studies around other regions of Brazil. Almeida et al. (2016) analyzed the sanitary and physiological quality of chia seeds from the commercial area of Pelotas, Rio Grande do Sul. The authors verified that four of the six evaluated lots showed the incidence of the fungi *Aspergillus* sp., *Bipolaris* sp., *Cladosporium* sp., and *Fusarium* sp., meanwhile, the percentages were low, between 0.5 and 1%. Herrera-Chan et al. (2019) evaluating the presence of fungi in chia seeds produced in the Tocantins identified five genera: *Cladosporium* sp., *Phoma* sp., *Fusarium* sp., *Alternaria* sp., and *Curvularia* sp..

The presence of these pathogens is not only concentrated in seeds produced or sold in Brazil. The species *Aspergillus niger*, and *A. Flavus*, besides the genera *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., and *Curvularia* sp. Were detected in seven seed samples from Paraguay (Jiménez et al., 2015). In a study performed by Njeri et al. (2019) to determine the level of contamination by fungi in seeds of chia cultivated and commercialized in Kenya verified the existence of *Rhizopus* spp., *Trichoderma* spp., and *Fusarium* spp..

It is worth emphasizing that even when incidence is low, as observed for the majority of the fungi encountered in chia seeds in this study, some species can be responsible for the appearance of an essential epidemics, thus, not having less importance because of the reduced occurrence and shall not be disregarded by the false impression of good quality of a seed lot. This is because, in addition to allowing the introduction of pathogens into farmland and cause diseases in crops, the presence of fungi on chia seeds can cause alterations in plant metabolism, compromising its therapeutic properties and flavor (Herrera-Chan et al., 2019).

Analyzing the ANOVA table, no interaction is present between the studied factors (lots x periods). However, *Phomopsis* sp. presented a significant difference to the evaluation periods and the other fungi presented for both factors separately (Table 1).

Table 1. Analysis of variance parameters for the incidence of *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Phomopsis* sp. identified in eight lots of saved chia seeds produced by farmers in the west of Parana state.

Source	DF	MS			
		Aspergillus sp.	*Penicillium* sp.	*Fusarium* sp.	*Phomopsis* sp.
Lot	7	1.1427*	0.9595*	3.5839*	0.6678^{ns}
Period	1	2.2350*	3.2761*	97.2442*	5.9597*
Lot X Period	7	8.1597^{ns}	0.2693^{ns}	20.764^{ns}	0.631^{ns}
CV (%)		31.2	28.1	23	44.4

Transformations: *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Phomopsis* sp.: square root of x+1

The mean values for contamination with the genera *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Phomopsis* sp. are presented in Table 2. It was observed that, for *Aspergillus* sp., lot 1 presented the highest incidence, even not statistically differing from lots 2, 4, 5, and 7. Lot 8 did not have the fungus incidence. In regards to *Penicillium* sp., a higher incidence was noted in lot 8, not significantly different; however, from lots 1, 2, 4, and 5. Both genera showed a similar behavior withing incubation periods, increasing their incidence from 7 to 14 days.

Variations in the fungi incidence in different chia seeds samples are probably due to weather conditions during their development, like precipitation during the maturation period and harvest or because of continuous cultivation of the same species or species of the same biological family, or due to storage (Herrera-Chan et al., 2019). These factors may influence the variations of the percentage of incidence observed among chia seed lots evaluated in this study since those lots are from different growers and consist of saved seeds that do not go through a rigorous production system during generations.

In a study with saved seeds of soybean, Bellé et al. (2016) found a high incidence of *Aspergillus* (above 60%) and *Penicillium* (40% average) at the seventh day of evaluation, which is higher than the values observed in this study that the highest average incidence was 5.5%. However, it does not mean that the pathogens cannot damage the seed lots. Both genera are storage fungi and occur in low-quality seeds, causing a deleterious effect in the seeds on the soil, or the death of plantlets after its establishment (Minuzzi et al., 2010).

For *Fusarium* sp., lot 8 had the highest incidence value (35% average), but without significant difference from lots 1, 2, 3, 4, and 6. Lot 7 presented the lowest fungi presence (14.25% average), differing only from lot 8. Similarly to the *Aspergillus* sp., and *Penicillium* sp., there was also a higher percentage of *Fusarium* sp. in the second period of evaluation (14 days of incubation). For the three genera at seven days of incubation, there was an incidence of fungi in the majority of the lots, except in lot 2 in the evaluation performed at seven days and lot 8 in both evaluation times, showing that it is the adequated period for their assessment via Blotter test. Even that lot eight did not show the incidence of *Aspergillus* sp., it showed the highest percentage of *Penicillium* sp., and *Fusarium* sp. Besides, *Fusarium* sp. was the fungi with the highest frequency in the present study. Herrera-Chan et al. (2019), from the five genera encountered in seeds of chia, *Fusarium* sp. was the second with higher incidence, only behind *Clamidosporium* sp.. Evaluating diseases in chia cultivation, Aguaysol et al. (2014), also verified the presence of *Fusarium* sp., *Rhizoctonia* sp., and *Sclerotinia sclerotiorum*, being the last one also observed González et al. (2010), differing from this study, in which the *Sclerotinia* sp. genus was not found.

Table 2. Incidence percentage of *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., and *Phomopsis* sp. identified in two incubation periods (days), in eight lots of saved chia seeds evaluated by the Blotter Test method.

Pathogen	Lots	Incubation periods		
		7	14	Average
Aspergillus sp.	1	2.0	4.0	3.00a
	2	0.25	2.5	1.38ab
	3	0.0	0.5	0.25b
	4	1.0	3.0	2.00ab
	5	1.5	2.0	1.75ab
	6	0.25	0.5	0.38b
	7	1.75	4.75	3.25a
	8	0.00	0.00	0.00b
	Average	0.84B	2.16A	
Penicillium sp.	1	2.5	4.75	3.63ab
	2	1.0	5.75	3.38ab
	3	1.0	2.75	1.88b
	4	2.75	5.0	3.88ab
	5	2.75	5.0	3.88ab
	6	2.25	2.25	2.25b
	7	0.75	2.00	1.38b
	8	5.50	5.50	5.50a
	Average	2.31B	4.13A	
Fusarium sp.	1	15.75	27.50	21.63ab
	2	5.75	39.75	22.75ab
	3	10.00	36.00	23.00ab
	4	13.00	35.25	24.13ab
	5	8.50	33.00	20.75b
	6	10.25	41.50	25.88ab
	7	4.25	24.25	14.25b
	8	30.25	39.75	35.00a
	Average	12.22B	34.63A	
Phomopsis sp.	1	0.00	1.25	0.63
	2	0.00	0.50	0.25
	3	0.00	3.75	1.88
	4	0.50	1.75	1.13
	5	0.25	0.25	0.25
	6	0.25	9.00	4.63
	7	0.50	1.75	1.13
	8	0.00	3.00	1.50
	Average	0.19B	2.66A	

*Averages followed by the same letter, uppercase in the column and lowercase in the row, do not statistically differ among themselves by the Tukey test at 5% of probability.

In regards to the *Phomopsis* sp. genus, there was a significant difference only between incubation periods, which can be observed the same tendency as for other genera that there is an increase in the percentage of incidence in the second evaluation period (14 days of incubation), about the first. This increase was expected because fungi pass through sporulation, and the contamination from one seed to another happens in the Petri dish. However, there was a different behavior for this fungus. Among the eight evaluated lots, half of them did not show the incidence of *Phomopsis* sp. at seven days of incubation. Still, after 14 days, all lots of seeds presented a percentage of the occurrence. It can be inferred that the referred fungus took more days to file mycelia growth and its development, thus, showing the importance of a more extended incubation period for its determination, avoiding a wrong interpretation for the presence of *Phomopsis* sp. in the seed lot. Differing from the other three discussed genera, a 14 days incubation period can be suggested for *Phomopsis* sp. as the best for its detection in chia seeds (Table 1).

Pereira et al. (2011), evaluating the incidence of *Phomopsis* sp. throughout the seed storage of soybean, observed a sharp reduction of the pathogen. This fungus rapidly loses viability when seeds are stored in natural environmental conditions. However, the presence of fungi in seeds can be related to the moisture, temperature, period of storage, and feed availability. These factors can favor the appearance and proliferation of fungi, which are responsible for the degradation of seeds and the production of (Carvalho et al., 2012; Rosseto et al., 2015). In this study, seeds were stored in a cold chamber with no moisture for four months before the analyses. However, the presence of *Phomopsis* sp. was identified.

About the percentage of transmissibility, it was observed that the fungus from the genus *Fusarium* sp., identified in lot 3, presented an index of 41%. Figure 1 illustrates the infection from the fungus in the aerial part (1ª) and the root system (1B) of plantlets. These results disagree with the ones from Herrera-Chan et al. (2019), in which, independently of the plant part that the fungi, including *Fusarium* sp., were inoculated, it was not observed pathogenic action and transmissibility.

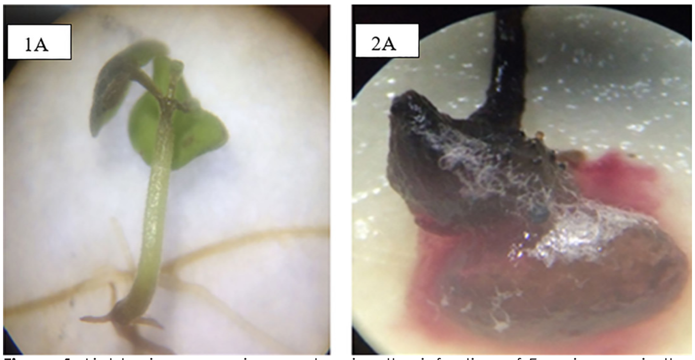

Figure 1. Light microscope image showing the infection of *Fusarium* sp. in the aerial part of the plantlet (1A) and rottenness in the root system (1B). (40x increase).

It is possible to observe the rottenness in the root system, a typical symptom of *Fusarium* sp. diseases. This fungus can also be associated with vascular wilt, and seed rot, besides of dumping-off (killing or weakness of seed or seedlings) (Kobayasti et al., 2011; Maciel et al., 2017). Agronomically, seeds contaminated by *Fusarium* sp. can cause significative losses (germination and vigor) in various crops due to the presence of the fungus in the propagation material, contamination, and soil persistence through chlamydospores, and the difficulty of decontamination when it is already established in the soil (Kobayasti et al., 2011).

The presence of various genera of fungi in the chia seeds evaluated in this study shows the importance of sanitary quality, avoiding the dissemination of pathogens to new areas. Identifying the different genera of fungi present in lots of saved seeds can help in taking control measures, avoiding their transmissibility to plantlets, and their presence in commercialized seeds for human consumption, with risk to affect human health because of the production of mycotoxins. The present study also demonstrates the importance of the correct period of incubation to determine the fungal incidence, considering that some genera, as observed for *Phomopsis* sp., can take a longer period to develop compared to the standard for Blotter test. That is important because it avoids a false interpretation of the test.

Conclusions

All evaluated seed lots of chia presented fungi contamination, and sixteen genera were detected, with a focus on *Fusarium* sp., *Phomopsis* sp., *Penicillium* sp., and *Aspergillus* sp.. The best evaluation period for *Phomopsis* sp. is at 14 days of incubation by the Blotter test method. The genus *Fusarium* sp. presents a high transmissibility rate from seed to plantlet.

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