Incidence of fungal species in stored soybean seeds in relation to cooling before packing and to packing material

Sanidade de sementes de soja refrigeradas e armazenadas: incidência de espécies fúngicas em sementes de soja armazenadas em função do resfriamento no ensaque e embalagens

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ABSTRACT

Maintaining seed quality during storage is a fundamental premise for production efficiency. The aim of this work was to evaluate the effect of cooling prior to packing seeds and of types of packaging on the occurrence of fungal species and the oscillations of these fungal populations over the storage period of soybean seeds. A completely randomized experimental design was used with four replications in a 2×3×5 factorial arrangement, consisting of cooling or no cooling of the seed mass before packing, three types of packaging materials, and five moments of evaluation during storage. Sanitary quality was evaluated by the method of incubation in filter paper without freezing. The seed mass cooled to 13°C prior to packing returned to ambient temperature in 15 days in a warehouse without controlled conditions. Cooling prior to packing does not affect the sanitary quality of soybean seeds during storage, whereas the type of packaging material does have an effect. The incidence of *Phomopsis* spp. and *Fusarium* spp. at the beginning of storage was lower mainly for seeds stored in the high-density polyethylene (HDPE) container. The occurrence of *Cercospora kikuchii* in both packaging types decreases over storage time. In all the packaging, the incidence of *Aspergillus* spp. and *Penicillium* spp. increases along the storage period.

Keywords: fungi, *Glycine max*, seeds quality, dynamic cooling.

RESUMO

A manutenção da qualidade das sementes durante o armazenamento é premissa básica à eficiência produtiva. O objetivo deste trabalho foi avaliar o efeito da refrigeração no pré-embalamento das sementes e dos tipos de embalagem sobre a ocorrência de espécies fúngicas e as oscilações dessas populações ao longo do armazenamento de sementes de soja. Utilizou-se o delineamento experimental inteiramente casualizado com quatro repetições, em arranjo fatorial 2x3x5 envolvendo ou não a refrigeração da massa de sementes antes do embalamento, três tipos de embalagens e cinco períodos de armazenamento. A qualidade sanitária foi avaliada por meio do método de incubação em papel de filtro sem congelamento. A massa de sementes resfriadas a 13°C no pré-embalamento retorna à temperatura ambiente em 15 dias, em armazém com condições não controladas. O resfriamento no pré-envase não influencia na qualidade sanitária das sementes de soja durante o armazenamento, já a embalagem afeta. Ocorreram reduções na incidência de *Phomopsis* spp. e de *Fusarium* spp. no início do armazenamento, principalmente para sementes armazenadas em contêiner de polietileno (PEAD). A ocorrência de *Cercospora kikuchii* em ambos tipos de embalagem decresceu ao longo do armazenamento. Em todas as embalagens, as incidências de *Aspergillus* spp. e de *Penicillium* spp. aumentam ao longo do período de armazenamento.

Palavras-chave: fungos, *Glycine max*, qualidade de sementes, resfriamento dinâmico.

INTRODUCTION

Various factors affect the quality of stored seeds, including the moisture content of seeds during storage, the use of packaging to conserve the seeds, the temperature and relative humidity of the storage environment, and the chemical composition of the seeds (Santos *et al*., 2016).

Quality control of soybean seeds is of fundamental importance within the scenario of constant technological evolution (Dode *et al*., 2013). This control is necessary to ensure the high quality of seeds in all their properties: physiological, physical, genetic, and sanitary. The sanitary aspect is relevant because seeds can be vehicles of plant pathogenic agents that may lead to germination reduction and plant vigor and may be the initial focal points of diseases. They may also cause damage, abortion, deformation, and rotting (Carvalho and Nakagawa, 2012).

Due to the increasing demand for seeds free of pathogens and of high physiological quality, seed production companies have sought new technologies not only to ensure germination and vigor after storage but also to guarantee seed health. Therefore, information on seed probable response to climatic conditions that occur during storage can assist decision making regarding storage of the product based on the cost-benefit ratio (Smaniotto *et al.*, 2014).

Maintaining seed quality during storage is essential to the soybean production chain because fields planted with high vigor soybean seeds tend to achieve better yields (Bagateli *et al*., 2019). Degradation of soybean seeds under storage is variable, depending both on the genetic traits of the soybeans and on the environmental conditions (Carvalho *et al*., 2014).

In this context, studies are necessary to evaluate and adapt technologies for storage of soybean seeds. The use of a modified atmosphere with the addition of carbon dioxide (CO_2), suitable packaging, temperature of the storage environment, and cooling of the seed mass before packing are among these technologies (Carvalho *et al*., 2016).

Artificial cooling of the seed mass at the packing time is an important alternative for conserving seeds in a conventional warehouse. The success of this technique is based on the possibility to maintain the initial temperature of the packaged seeds at safe levels (Demito and Afonso, 2009).

The combined use of product cooling and monitoring of moisture content is important for fungal proliferation control (Scussel, 2002). The use of artificial refrigeration directly on the seed through injection of cold air at the time of packing allows the maintenance of physiological quality during the storage period, decreasing fungal infection (Baudet, 2003). Zuchi and Bevilaqua (2014) did not observe any effects from dynamic artificial cooling on soybean seed health. The relationship between physiological quality and artificial cooling by the dynamic method is controversial, and contrasting results remain (Zuchi *et al*., 2013; Virgolino *et al*., 2016). Clarification is needed regarding the relationship between the temperature of the seed mass and the response of the fungal population over the storage period of soybean seeds.

The use of suitable packaging during storage is essential for maintaining seeds physiological quality. The package types, depending on their properties, can reduce or impede water vapor exchanges between the seeds and the outer environment, maintaining the initial moisture content of the seeds (Bessa *et al*., 2015). Packages that hold larger volumes, near 1000 kg, together with cooling of the seed mass, constitute promising options (Carvalho *et al*., 2016; Virgolino *et al*., 2016).

Thus, the aim of this study was to evaluate the effect of cooling prior to packing of seeds and packaging types on the occurrence of fungal species and fluctuations in fungal populations during storage of seeds under non-climate-controlled conditions.

MATERIALS AND METHODS

The soybean seeds used in this study were from the cultivars TMG 1176 RR and SYN 9074 RR; the seed lots were produced by Grupo AMAGGI, under similar conditions, in Campo Novo do Parecis city, Mato Grosso (MT) state, Brazil. The municipality has an Aw type climate (tropical savanna, with higher concentration of rain in the summer) according to the Köppen classification, with mean annual rainfall of 1945 mm and mean annual temperature of 23.7°C (Souza *et al*., 2013). The seed lots used had similar initial characteristics regarding germination and vigor. All the seed lots were processed following the same procedures.

The seeds were dried in a stationary forced air circulation dryer at 38°C until 12% moisture content was reached. Part of the seed mass was cooled artificially until reaching 13°C, using an appropriate air-cooling device (PCS 80 - CoolSeed®), and the other part was not cooled before packing. The seeds were placed in three types of packages: multi-layered "kraft" paper (40 kg capacity), woven polypropylene ("big bags", 1000 kg capacity), and molded high-density polyethylene containers (HDPE, with lid, 1000 kg capacity) and then stored. After packing, the seeds were stored for eight months in a conventional storage unit (non-climate-controlled conditions) in Campo Novo do Parecis. Temperature in the packaging was monitored weekly, with rod thermometer 100 cm (Czaki Thermo Product), at a depth of 50 cm in the seed mass, always at the same time of the day, 11:00 a.m., with four replications.

Evaluations of seed sanitary quality were performed over the storage period at 0, 2, 4, 6, and 8 months of storage. Sanitary analyses were performed in the Seed Pathology Laboratory of UFLA, Lavras city, Minas Gerais (MG) state, Brazil. The method of incubation in filter paper without freezing was used (Neergaard, 1979). Eight replicates of 25 seeds per sample were incubated in the laboratory. The seeds were distributed on 15-cm-diameter Petri dishes containing three sheets of filter paper moistened with water and sodium dichlorophenoxyacetate (2,4-D) at 10 ppm, agar diluted in 10 g of the product and 990 ml of water, all materials sterilized in autoclave at 120 ºC for 30 minutes. The Petri dishes were kept in an incubation room at 20ºC with a photoperiod of 12 hours, where they remained for seven days, after which they were evaluated for the presence of fungi (Brasil, 2009). Seeds were individually examined through of a magnifying glass and an optical microscope, identifying the fungal species through the typical structures of the fungi formed in the seeds. The results were expressed as a percentage of occurrence of fungi.

A completely randomized experimental design (CRD) was used with four replications in a 2 × 3 × 5 factorial arrangement consisting of cooling or no cooling of the seed mass before packing, three types of packaging, and five moments of evaluation during storage (0, 2, 4, 6, and 8 months). Analyses were performed independently for each cultivar. To meet statistical assumptions, the data were first transformed in $(x + 1)^{1/2}$. Analysis of variance was carried out with the assistance of the Sisvar® software (Ferreira, 2014) at 5% probability by the F test. The mean values were compared by the Tukey test at 5%, or polynomial regression analyses were carried out with the choice of mathematical models significant at 5%, with higher value of coefficient of determination and biological relation.

RESULTS AND DISCUSSION

Temperature of the seed mass

After cooling to 13°C prior to packaging, the temperatures of seed mass of the cultivar TMG 1176 RR, regardless the type of package type, returned to the level above 20°C after one week of storage under non-controlled conditions (Figure 1a). A similar tendency was found for the cultivar SYN 9074 RR; however, with lower temperature values up to the second week of storage for seeds cooled prior to packaging and then placed in larger volumes packages , the big bag and polyethylene container (Figure 1b).

Virgolino *et al*. (2016) reported that after cooling, the soybean seed mass maintained lower temperature for a longer period in greater capacity packages (big bags – 1000 kg). Low temperatures reduce cell respiration, retard the deterioration process, and inhibit insect and microflora activity (Smaniotto *et al*., 2014).

The temperatures of the seed mass, cooled or not prior to packing, had similar values, approximately 22°C, after 3 weeks of storage, regardless the type and volume of the package (Figure 1). Greater oscillations occurred in the initial months of

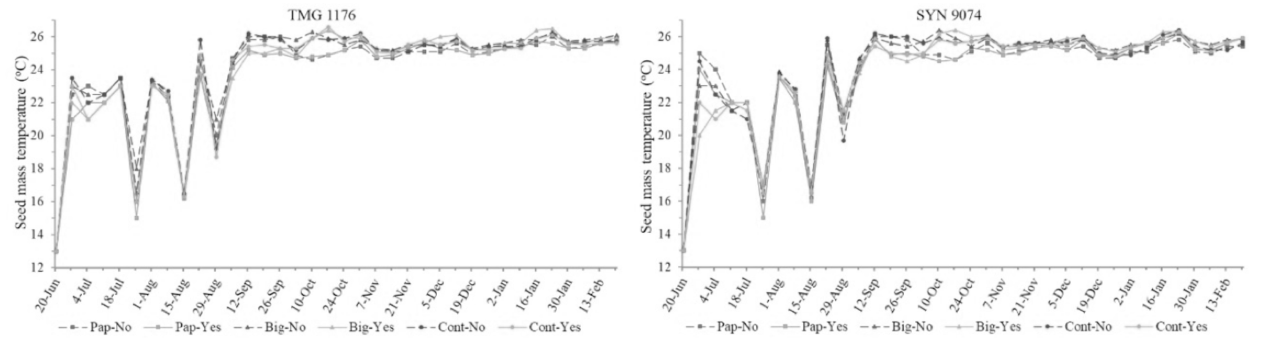


Figure 1 - Temperature of the seed mass, at 50 cm depth, from cultivars TMG 1176 and SYN 9074 not cooled (No) or cooled (Yes) before packaging and storage in different package types (Paper bag - Pap, Big Bag - Big, Container - Cont), during storage.

storage, June, July, and August; after that period, the temperatures of the seed masses remained higher, in general above 22°C, with small oscillations in the other months (Figure 1). Variations occurred in accordance with the temperature of the warehouse environment, which was not controlled and oscillated in accordance with the climate conditions of each season of the year in the region. Zuchi *et al*. (2013), also with storage in the state of Mato Grosso, reported lower temperatures in the seeds cooled only in the first fifteen days of storage in a warehouse with non-controlled conditions.

Sanitary quality of the seeds

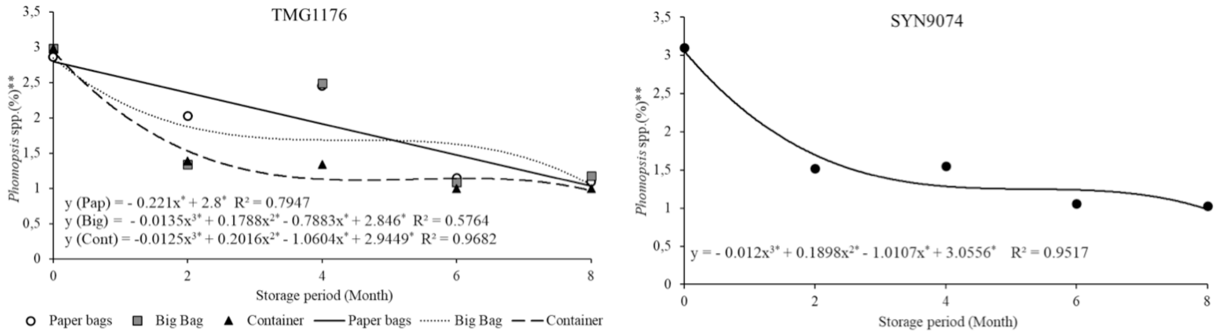
In the seed health tests, the following fungi were detected: *Cercospora kikuchii* (*kikuchii* (Tak. Matsumoto & Tomoy.) M.W. Gardner), *Phomopsis* spp., *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp.

For the cultivar TMG 1176, the package types and storage factors had significant interaction with the incidence of *C. kikuchii* and *Phomopsis* spp., and triple interaction (package types, cooling, and storage) for *Penicillium* spp. For the incidence of *Fusarium* spp., the isolated package types and storage factors showed a significant effect; the latter factor also affected the occurrence of *Aspergillus* spp. The values of the coefficients of variation ranged from 24.5% for *Penicillium* spp. to 33.6% for *Aspergillus* spp.

For the cultivar SYN 9074, there was significant interaction of the package types and storage factors for the incidence of *C. kikuchii*. The storage factor was significant for *Phomopsis* spp., *Fusarium* spp., *Aspergillus* spp. and *Penicillium* spp. The coefficients of variation ranged from 26.5 % for *Phomopsis* spp. to 36.6% for *Aspergillus* spp.

The occurrence of the fungi *Phomopsis* spp. in seeds of the cultivar TMG 1176 stored in the container or big bag tended to exhibit a third-degree effect, with sharp reduction in the first two months of storage (Figure 2a). After that period, there was a tendency toward stabilization at low real values, not more than 3%, and reduction in the final months, with actual values near 0% incidence. A similar tendency was also observed for seeds of the cultivar SYN9074, regardless of the package types (Figure 2b). The seeds of TMG 1176 stored in paper bags exhibited a linearly inversely proportional effect, with reduction in the incidence of *Phomopsis* spp. as the storage period proceeded (Figure 2a).

A reduction was found in the incidence of *Phomopsis* spp. in the seeds during the storage period in all the packages used. This fungus is more common in the field (Henning, 2005). *Phomopsis* spp. rapidly loses viability during the storage period and thus its incidence is reduced. Storage of seeds in packages with larger volumes, big bag and polyethylene containers, led to lower incidences of *Phomopsis* spp. at the beginning of storage (2 months), an effect that continued for seeds stored in the polyethylene container at 4 months, which showed a lower incidence (Table 1).



**Data transformed in (x+1)½.

Figure 2 - Incidence of *Phomopsis* spp. (%) in the soybean seeds from cultivars TMG 1176 stored in different package types and SYN 9074 and after different storage periods.

Table 1 - Incidence of *Phomopsis* spp. (%) in the soybean seeds from cultivar TMG 1176 stored in different package types and after different storage periods

Package type	TMG 1176				
	Storage period (month)				
	0	2	4	6	8
Paper Bag	7,5 a	3,8 b	5,8 b	0,5 a	0,3 a
Big Bag	8,3 a	1,0 a	5,5 b	0,3 a	0,5 a
Container	8,3 a	1,3 a	1,0 a	0,0 a	0,0 a

*Means followed by the same letter in the column do not differ between each other according to the Tukey test at 5%. The original means were presented, but the data were compared according to the data transformed in (x+1)½.

The incidence of *Fusarium* spp. over the storage period had a similar tendency to that found for *Phomopsis* spp. (%) for both cultivars, regardless the package type and the cooling (Figure 3). It exhibited sharp reduction in the first 2 months and then tended to stabilize at low incidences, at actual values near 3%, above all at the end of the storage period (8 months). An explanation for this is that it is a fungus that commonly occurs in the field; according to Pereira *et al.* (2007), fungi from the field decline in infestation in seeds during the storage period. The fungi from the field, *Phomopsis* spp. and *Fusarium* spp., decline in incidence over the storage period because they lose their ability to infect (Henning, 2017).

Just as was found for *Phomopsis* spp., the type of package affected the incidence of *Fusarium*

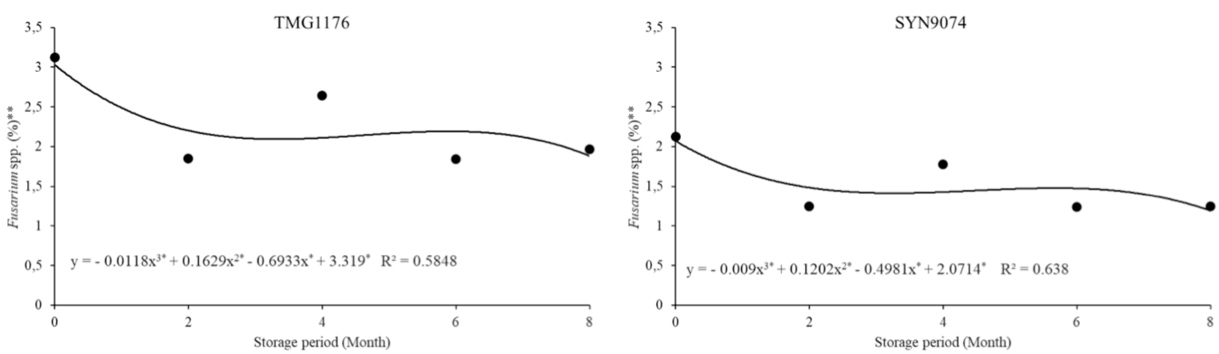


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Figure 3 - Incidence of *Fusarium* spp. (%) in the soybean seeds from cultivars TMG 1176 and SYN 9074 after different storage periods.

Table 2 - Incidence of *Fusarium* spp. (%) in the soybean seeds from cultivar TMG 1176 stored in different package types

Package type	Fusarium spp. (%)
Paper Bag	5,8 b
Big Bag	5,3 b
Container	3,8 a

*Means followed by the same letter in the column do not differ between each other according to the Tukey test at 5%. The original means were presented, but the data were compared according to the data transformed in $(x+1)^{½}$.

spp. In seeds of the cultivar TMG 1176, a lower occurrence of *Fusarium* spp. was found in seeds stored in molded high density polyethylene containers (HDPE, with a lid, 1000 kg capacity) (Table 2). This emphasizes the importance of the choice of the ideal type of package that is unfavorable to the proliferation of fungi and thus maintains sanitary quality and, consequently, physiological quality.

Seeds of the cultivar TMG 1176 stored in paper bags showed a sharp decline in the incidence of *C. kikuchii* in the first 2 months of storage, reaching actual values near 0 (Figure 4a). For seeds stored in the polyethylene container, reduction in the incidence of *C. kikuchii* occurred only after 2 months of storage, with prominent reduction up to 6 months. Seeds stored in big bags showed sharp reduction up to 4 months of storage, with a minimum value of incidence (approximately 1% in transformed value, equivalent to 0% in actual value) reached at 6.58 months of storage; after that period the values remained near 0% (Figure 4a).

C. kikuchii is also a fungus that typically occurs in the field, and thus its incidence declines over a storage period. Some authors have reported no negative effect of this fungus on embryonic tissues, and thus no effect on germination (Feliceti *et al*., 2018). However, this pathogen is a causal agent of disease in the field.

For the cultivar SYN9074, sharp declines in the incidence of *C. kikuchii* were found initially and continued until 4 months of storage in seeds stored in big bags and in polyethylene containers, with minimum values (0% in actual values) obtained at 6.87 and 6.95 months of storage in big bags and polyethylene containers, respectively (Figure 4a). For seeds stored in paper bags, incidence declined considerably after 2 months of storage, with low occurrences after 4 months with values near 0%.

The package types differed in regard to the occurrence of *C. kikuchii* only in the initial periods of storage, 0 and 2 months (Table 3). As the storage period proceeded after 4 months, the incidences were near 0%, regardless the package type.

For seeds of the cultivar TMG 1176, there was an increase in the incidence of the fungus *Aspergillus* spp. at the beginning of storage, from 0 to 2 months. However, there was a sharp increase after 6 months, rising to 1.3% at 6 months and to 7.5% at eight months (Figure 5a). For seeds of the

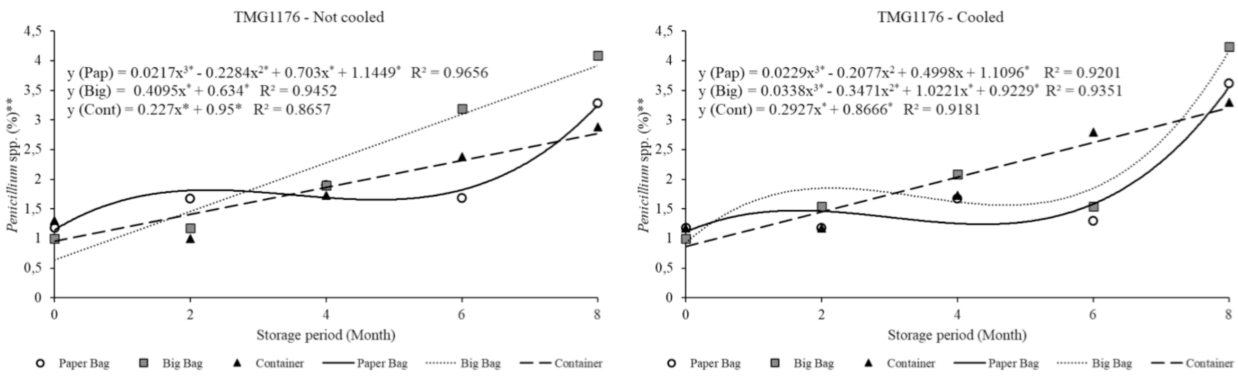

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Figure 4 - Incidence of *Penicillium* spp. (%) in the soybean seeds from cultivar TMG 1176 not cooled or cooled before packaging and stored in different package types and after different storage periods.

Table 3 - Incidence of C*ercospora kikuchii* (%) in the soybean seeds from cultivars TMG 1176 and SYN 9074 stored in different package types and after different storage periods

Package type	TMG 1176					SYN 9074				
	Storage period (month)					Storage period (month)				
	0	2	4	6	8	0	2	4	6	8
Paper Bag	7,0 b	0,5 a	0,5 a	0,0 a	0,0 a	3,7 a	6,0 b	0,2 a	0,2 a	0,0 a
Big Bag	5,5 b	2,7 b	0,5 a	0,0 a	0,0 a	6,2 b	3,2 a	0,5 a	0,0 a	0,0 a
Container	2,0 a	3,1 b	0,0 a	0,0 a	0,0 a	5,0 ab	2,0 a	0,5 a	1,0 a	0,0 a

*Means followed by the same letter in the column do not differ between each other according to the Tukey test at 5%. The original means were presented, but the data were compared according to the data transformed in $(x+1)^{1/2}$.

cultivar SYN 9074, the incidence of *Aspergillus* spp. and the period of storage had a direct linear relationship; the longer the period of storage, the higher the incidence, regardless of the package type and cooling prior to packing (Figure 5b). Since *Aspergillius* spp. is a storage fungus, its incidence rates increase over the period of storage of soybean seeds (Cardoso *et al*., 2004). According to Carvalho *et al*. (2014), after 6 months of storage, the quality of soybean seeds declines. Higher values of electrical conductivity imply greater release of exudates to the medium through the membrane, indicating more extensive destructuring of the membrane. This may be related to a greater incidence and colonization of storage fungi in the seeds.

The occurrence of *Penicillium* spp. (%) in seeds of the cultivar TMG 1176 not cooled prior to packing (Figure 6a) generally increased over the storage period in a direct linear manner for seeds stored in big bags and polyethylene containers. For those stored in paper bags, there was an initial increase in the incidence of the fungi in the first 2 months and a sharp increase after 6 months of storage. This tendency was also found for seeds cooled before packing and stored in paper bags and big bags (Figure 6b), with a prominent rise in the incidence of fungi after 6 months of storage. For seeds cooled and then stored in the polyethylene container, just as for the non-cooled seeds, the incidence of *Penicillium* spp. increased as the storage period advanced.

In the cultivar SYN 9074, the incidence of *Penicillium* spp. on the seeds and the period of storage had a direct linear relationship (Figure 7); the incidence of *Penicillium* spp. increased progressively throughout the storage period, regardless of the package type and cooling prior to packing. Since it is a storage fungus, the incidence rates tend to increase over the storage period of the seeds. Soybean genotypes have different levels of tolerance to storage (Carvalho *et al*., 2014), which may be related to the different responses and pathogen incidence levels in the seeds of the two cultivars evaluated. However, studies are necessary to clarify this possible relationship.

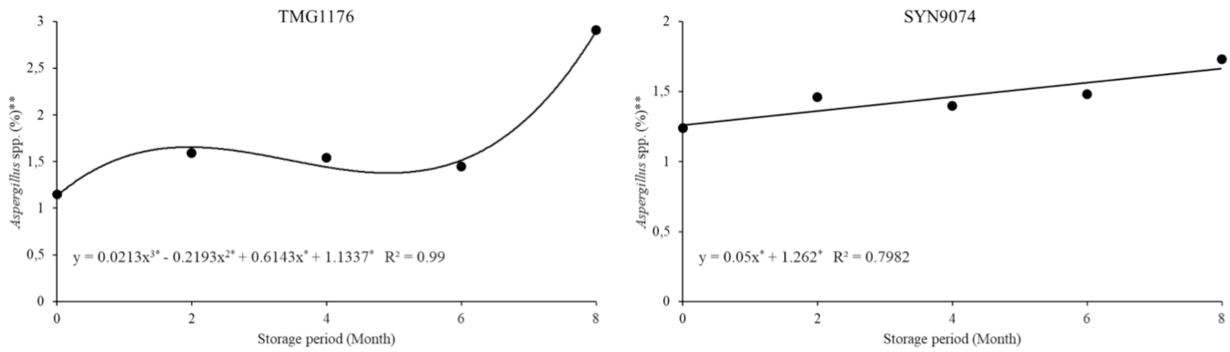

Figure 5 - Incidence of *Aspergillus* spp. (%) in the soybean seeds from cultivars TMG 1176 and SYN 9074 after different storage periods (0, 2, 4, 6, and 8 months).

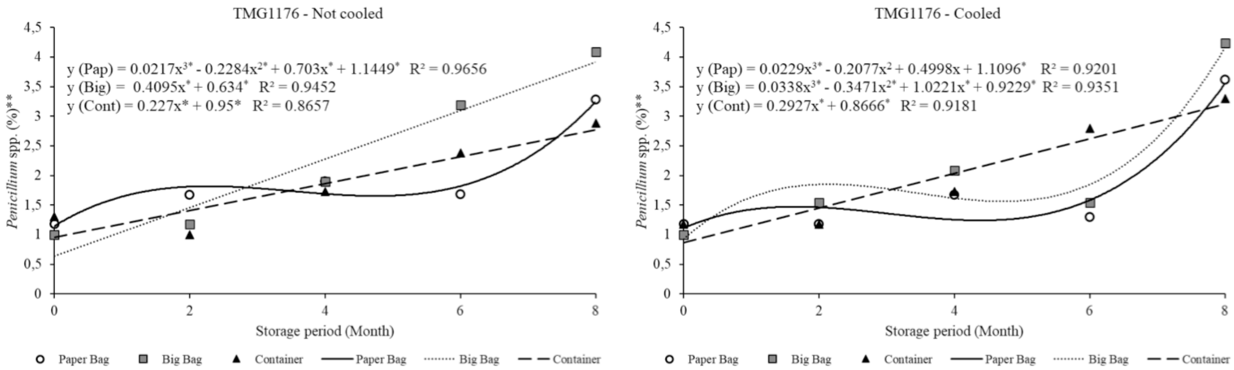

Figure 6 - Incidence of *Penicillium* spp. (%) in the soybean seeds from cultivar TMG 1176 not cooled or cooled before packaging and stored in different package types and after different storage periods.

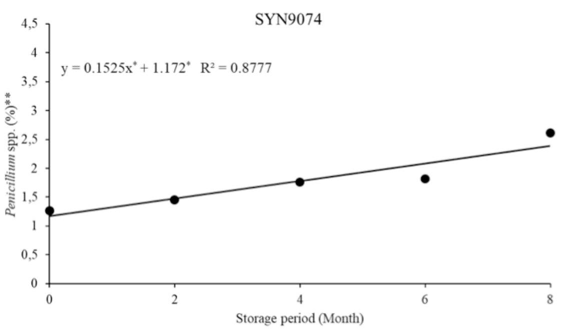

Figure 7 - Incidence of *Penicillium* spp. (%) in the soybean seeds from cultivar SYN 9074 after different storage periods (0, 2, 4, 6, and 8 months).

The incidences of *Penicillium* spp. on the seeds of the cultivar TMG 1176 were low at the beginning of storage, and there was no difference between package types or between the application of cooling or not, up to 4 months. Values were very near 0% in the first 2 months (Table 4). As storage time advanced, at 6 and 8 months, seeds stored in big bags had a higher incidence of *Penicillium* spp., except for those cooled at 6 months, in which the highest value was found in seeds stored in the polyethylene containers.

The cooling prior to packing only led to a lower incidence of *Penicillium* spp. in seeds packed in big bags and evaluated at 6 months of storage (Table 4). Cooling of the seed mass did not ensure improvements in sanitary quality of seeds and did not reduce the incidence of pathogens during storage. This is related to the return of the temperature of the seed mass to the temperature of equilibrium with the environment in the initial

Table 4 - Incidence of *Penicillium* spp. (%) in the soybean seeds from cultivar TMG 1176 not cooled (NC) or cooled (C) before packaging and stored in different package types and after different storage periods

Package type	TMG 1176									
	0		2		4		6		8	
	NC	C	NC	C	NC	C	NC	C	NC	C
Paper Bag	0,5 a	0,5 a	2,0 a	0,5 a	3,0 a	2,0 a	2,5 a	1,0 a	10,0 ab	12,5 ab
Big Bag	0,0 a	0,0 a	0,5 a	1,5 a	3,0 a	3,5 a	9,25 bB	1,5 aA	16,0 b	17,5 b
Container	1,0 a	0,5 a	0,0 a	0,5 a	2,0 a	2,0 a	5,0 ab	7,0 b	7,5 a	10,0 a

*Means followed by the same lower case letter in the column do not differ between each other according to the Tukey test at 5%. Upper case letters in the line, in each storage period, present significant differences according to the variance analysis, p<0.05 by the F test. The original means were presented, but the data were compared according to the data transformed in $(x+1)^{1/2}$.

weeks of storage and the fact that other cycles or forms of cooling afterwards were not performed. The lower temperature of the seed mass for only 2 weeks was not sufficient to alter the incidence of fungi. Zuchi *et al.* (2013) also observed thermal equilibrium of cooled seeds with the temperature of the warehouse in 15 days. For Zuchi and Bevilaqua (2014), dynamic cooling did not reduce the incidence of *C. kikuchi*, *Fusarium* spp., and *Phomopsis* spp.

CONCLUSIONS

The seed mass cooled to 13°C prior to packing returns to ambient temperature in 15 days in a warehouse without climate control in a region with Köppen climate classification of Aw (tropical savanna, with highest concentration of rains in the summer).

Cooling prior to packing does not have an effect on the sanitary quality of the soybean seeds during storage.

The package type affects the incidence of fungal populations in soybean seeds over the storage period.

Reductions occur in the incidence of *Phomopsis* spp. and *Fusarium* spp. at the beginning of storage, 2 months, especially for soybean seeds stored in the polyethylene container (HDPE).

The occurrence of *Cercospora kikuchii* decreases over the storage period of soybean seeds, with a sharp reduction at 4 months.

In all the package types, the incidences of *Aspergillus* spp. and *Penicillium* spp. increase as the storage period of soybean seeds advances, above all after 6 months.

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