Scientific Paper

Fungal population associated to the germination process of stored seeds of Leucaena leucocephala *cv. Peru*

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ABSTRACT: The objective of this research was to identify the fungi associated to stored seeds of *Leucaena leucocephala* cv. Peru, during the germination test. For such purpose a mycological analysis was performed on seeds with 6 and 18 months of storage (400 in each case, in four replications of 100), from the storehouse of La Rioja seed farm –belonging to the Livestock Production Enterprise Martí (Matanzas province, Cuba)–. The fungal agents were identified with the aid of taxonomic keys, and the data related to the number of germinated seeds, seeds with disease symptoms and seeds rotten by fungi were processed through the system of comparison of proportions. Eleven fungal species were diagnosed: *Penicillium expansum*; *Aspergillus flavus,*isolates 2, 3 and 4; *Aspergillus niger*; *Fusarium oxysporum*; *Trichoderma* sp.; *Pestalotia* sp.; *Rhizopus stolonifer*; *Cladosporium sphaerospermum* and *Aspergillus wentii*. Among them the first one stood out, with a maximum of 11,25 % of infection in the rotten 18-month seeds. The main symptoms were: seed discoloration, spots on the cotyledons and necrosis of the radicle. It is concluded that the fungal species found belong to seven of the most representative genera, which come from the field and the storehouse. The fungal agent *P. expansum* showed the highest infection percentage in the germinated and rotten seeds. Only six of the 11 species of fungi caused the main detected symptoms (*P. expansum*, *A. flavus*isolate 2, *A. niger, F. oxysporum*, *Trichoderma* sp. and *Pestalotia* sp.

Keywords: forage legumes, fungi, seed storage

INTRODUCTION

Stored seeds constitute an important alternative in the plant cultivation programs of a country, and represent an essential link with the successive generations. However, as any living organism, they can be affected by many factors, such as temperature, humidity, pressure of oxygen, as well as rodents, pest insects and pathogens; which, in close interrelation, deteriorate them and decrease their vigor and quality (Doria, 2010).

According to Ridao (2003), the association of pathogens with the seeds causes not only direct losses of the plant populations in the field, but also irreversible effects in a whole agricultural system. For a large number of devastating diseases, the seed represents the ideal vehicle of dissemination and perpetuation of their causative agents, and it is where the inoculant source, which is capable of infesting the new crop that is planted, persists. Hence the importance of evaluating the health factor, together with the indicators germination percentage, purity and vigor, to determine the quality of a seed lot.

However, no thorough studies have been conducted on the stored seeds of *Leucaena leucocephala* cv. Peru, for which the available information on the topic is scarce and insufficient in Cuba and worldwide. Nevertheless, the specialized literature reports that *Alternaria*, *Aspergillus*, *Botrytis*, *Cladosporium*, *Curvularia*, *Doratomyces*, *Fusarium*, *Helminthosporium*, *Macrophomina*, *Nigrospora*, *Penicillium*, *Chaetomium*, *Pestalotia* and *Rhizopus* represent the fungal genera most associated to such seeds under ambient storage conditions (Alonso *et al*., 1996; Lezcano *et al*., 2007).

In addition, Lezcano *et al*. (2007) also detected diverse disease symptoms in the seeds of this legume, such as: discoloration of the seed coats, rot, spots on the cotyledons and death of the radicles, which cause the significant reduction of their germination and the loss or decrease of their viability. For such reason, it is necessary to conduct studies on the most important microflora associated to these seeds, to determine its possible control.

The objective of the research was to identify the main fungi associated to the stored seeds of *L. leucocephala* cv. Peru, during the germination test.

MATERIALS AND METHODS

The study was conducted in the plant protection laboratory of the Pastures and Forages Research

Station Indio Hatuey –Matanzas, Cuba–, and in the plant mycology laboratory of the National Center of Agricultural Health –Mayabeque, Cuba.

Origin of the samples. The samples, collected in the seed storehouse of the La Rioja seed farm –belonging to the Livestock Production Enterprise Martí, Matanzas province, Cuba–, were germinated (GS) and rotten seeds (RS) of *L. leucocephal*a cv. Peru, with 6 and 18 months of storage under ambient conditions, which showed signs of fungal agents or symptoms of their diseases; they were detected during the germination test.

Germination test and procedure for the identification of the fungi. The germination test was carried out according to the recommendations made by ISTA (1999) for tropical trees, and the germination counts were made every three days. A completely randomized design was used; the treatments were represented by the storage times, and for each one 400 seeds were used (100 per replication). They were distributed in four glass Petri dishes –sterile, 15 cm of diameter– which contained two sheets of filter paper –also sterile– that were used as inert substratum, and other two used for covering them, to which no treatment was applied. During the experiment (21 days), the dishes were maintained in incubation, under controlled conditions of light (full darkness) and temperature (25 ºC as average).

The seeds that showed signs of fungi or symptoms of diseases were removed. Then the causative agent was isolated (with the aid of previously disinfected needles, and of a Zeiss SV-6 stereoscopic microscope –ocular lenses from 10 to 100 x–), the pure cultures were obtained and, finally, they were identified to the genus and/or species level, according to Martínez *et al*. (1992) and ISTA (1999).

The keys used for the identification of the fungi, according to the cultural and morphological traits, were the ones proposed by: Raper and Fennell (1965), Onions (1966a, 1966b, 1966c), Booth (1969), Rifai (1969), Ellis (1971), Lunn (1977), Sutton (1980), Barnett and Hunter (1999), Ho *et al*. (1999) and López (2003).

Measurements. The infection percentage caused by fungi in the GS and RS with 6 and 18 months of storage was calculated; for that purpose the total GS and RS in each storage time (284 and 104 for those of 6 months; 19 and 122 for the ones with 18 months) was taken into consideration. Likewise, the number of germinated seeds with symptoms of fungal diseases or signs of these agents (GSWSD) and the number of seeds rotten by fungi (RS) were measured.

Statistical analysis. The data related to the infection of the fungal agents on the seeds during germination were processed through the system of comparison of proportions –version 2.1– (CENSA, 1998).

RESULTS AND DISCUSSION

In the seeds of *L. leucocephala* cv. Peru with 6 and 18 months of storage under ambient conditions, 11 fungal agents were identified, belonging to the genera: *Penicillium*, *Aspergillus*, *Fusarium*, *Rhizopus*, *Pestalotia*, *Cladosporium* and *Trichoderma*.

Table 1. Fungal infection in seeds of *L. leucocephala* cv. Peru with 6 months of storage.

Fungus	GSWSD (%)	RS (%)
P. expansum	5,60[a]	5,27[a]
A. niger	5,60[a]	–
A. flavus isol. 2	4,93[ab]	0,53[c]
F. oxysporum Schltdl	3,52[b]	1,58[b]
R. stolonifer Ehrenb. ex Fr.	3,52[b]	–
Pestalotia sp.	1,05[c]	–
Trichoderma sp.	1,05[c]	0,53[c]
A. flavus aisl. 3	–	1,05[bc]
C. sphaerospermum Penz	–	2,63[b]
SE ±	0,01	0,08

Different letters in the columns differ at $p \leq 0,001$.
GSWSD: germinated seeds with signs of fungi and symptoms of their diseases, RS: seeds rotten by fungi

Table 1 shows the microflora associated to the seeds with 6 months of storage. The fungi *Penicillium expansum* Link and *Aspergillus niger* van Tieghem were the ones that infected the most the GSWSD; although the infection percentage of *Aspergillus flavus* isolate (isol.) 2 did not differ from these two fungal agents. In the case of RS, *P. expansum* also caused the highest affectation, and the statistical difference was higher with regards to the other fungi.

It is important to state that the infection in the GSWSD was higher, due to the existence of a higher quantity of susceptible plant tissue (seed coats, cotyledonal leaves and radicles) which was exposed to the action of fungi (fig 1a); while in the RS, the infection was localized only on those that did not germinate or on their coats (fig. 1b). On the other hand, the values of seed rot did not exceed 6 % of infection, which also confirmed the scarce affectation produced by fungi.

Regarding the fungal population associated to the seeds of *L. leucocephala* cv. Peru with 18 months of storage (table 2), the fungus *P. expansum* caused the most significant infection percentage in the GSWSD as well as in the RS, and differed significantly from the other fungal agents. In addition, the highest fungal affectation occurred in the RS (fig. 2), with presence of a higher quantity of species; among them *Aspergillus flavus*isol. 4 and *Aspergillus wentii* Wehmer, which were not previously reported in GSWSD or in the seeds with 6 months of storage.

The increase of the microflora present in the RS (table 2) showed that the infection increased with the passing of the storage time, because higher multiplication, reproduction and growth of the following species were observed: *P. expansum, A. flavus* isol. 2, *A. flavus* isol.3, *Rhizopus stolonifer*, *Cladosporium sphaerospermum* and *Trichoderma* sp., which favored the accelerated deterioration and death of the infected seeds.

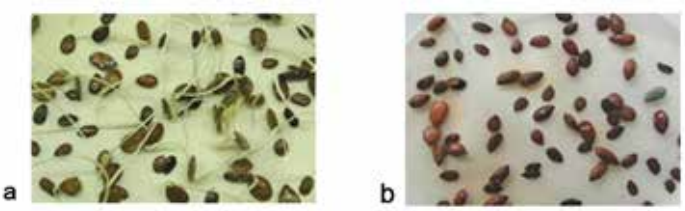

Figure 1. Germinated seeds with symptoms of disease (a) and rotten seeds with 6 months of storage (b).

Table 2. Infection caused by the fungi associated to the seeds with 18 months of storage.

Fungal agent	GSWSD (%)	RS (%)
P. expansum	3,25[a]	11,25[a]
A. flavus isol. 2	1,75[b]	2,75[b]
A. flavus isol. 4	–	2,25[bc]
A. flavus isol. 3	–	1,75[c]
A. niger	1,75[b]	1,75[c]
Rhizopus stolonifer	–	3,75[b]
C. sphaerospermum	–	2,75[b]
F. oxysporum	1,25[b]	1,25[cd]
A. wentii	–	0,50[d]
Trichoderma sp.	0,50[c]	1,50[cd]
SE ±	0,02	0,03

Different letters in the columns differ at a $p \leq 0,001$.
GSWSD: germinated seeds with signs of fungi and symptoms of their diseases, RS: seeds rotten by fungi

Figure 2. Rotten seeds with 18 months of storage.

According to Schmidt (2000), the fungi that belong to the genera *Penicillium* and *Aspergillus* constitute the main agents that affect orthodox seeds under storage conditions; this category includes the seeds of the *Leucaena* genus. Soetrismo (2003) stated that, together with the species of *Rhizopus*, this is the most common microflora in stored seeds, particularly in the *L. leucocephala* seeds. These results coincide with the ones obtained by Sahu and Agarwal (2004), who also reported for this legume the presence of the species: *A. flavus, A. niger, Alternaria alternata, Alternaria tenuissima, Apiospora montagnei, Arthrinium euphorbiae, Aspergillus fumigatus, Curvularia clavata, Curvularia lunata* [*Cochliobolus lunatus*], *Fusarium* sp., *Fusarium pallidoroseum* (syn. *Fusarium semitectum*), *Paecilomyces variotii, Penicillium chrysogenum* and *Phoma* sp.

Another important aspect was the remarkable increase of the concentration of lixiviated solutes in the rotten seeds with 18 months of storage, due to the damage caused by the microorganisms to the tissues and, particularly, to the cell membrane; which was more evident in the seeds affected by *Aspergillus* (*A. flavus*), *Penicillium*, *Cladosporium* and *Fusarium*. According to Halloin (1986), in general this damage is direct or indirect consequence of the presence of extracellular enzymes of the microorganisms (cellulases, pectinases, lipases, proteases and nucleases) and their toxins, which are also responsible for the inhibition of the normal growth of seedlings in many crops (Howlett, 2006). Besides, Latiffah *et al*. (2014) state that the establishment of fungi on the seeds disturbs their metabolic activity, and the nutritive tissues are consumed by fungal microorganisms, which causes viability to decrease. Likewise, discoloration and loss of the germination capacity can be appreciated in the seeds (FAO, 2010).

Undoubtedly, the species that were identified represent the most important microflora associated to the *L. leucocephala* seeds, which coincides with the report by Alonso *et al*. (1996).

In the specific case of the infection by *Fusarium*, the report by Borges and Urdaneta (2010) was confirmed, regarding the fact that this agent reduces germination and causes seedling rot and death. Such results coincide with the results obtained by Gally *et al*. (2006) about the fact that *Fusarium* spp. and *Phomopsis* spp. increase the number of dead seeds and affect the emergence of soybean (*Glycine max* L.) seedlings.

There are diverse reports about the incidence of *P. expansum*, *A. flavus*, *A. niger*, *R. stolonifer* and *Fusarium oxysporum* associated to seeds of many plant species worldwide, such as: *Moringa oleifera* Lamarck, *Jatropha curcas*, *Vigna unguiculata* L. Walp., *Cajanus cajan* L., *Phaseolus vulgaris* L., *Arachis hypogea* L., *G. max* and *Zea mays* (Jayaraman *et al*., 2011; Rathod *et al*., 2012; Costa *et al*., 2013; Ghangaokar and Kshirsagar, 2013; Martínez *et al*., 2013; Martínez *et al*., 2014; Rajuskar and Taware, 2014).

It is important to stress that there was a close relation between the disease symptoms and the microflora, because the almost direct link of the seed discoloration with the incidence of *P. expansum*, *A. niger*, *Pestalotia* sp., *Trichoderma* sp., *A. flavus* isol. 2 and *F. oxysporum* was observed; as well as the presence of spots on the cotyledons and the necrosis of the radicles caused by *A. flavus* isol. 2 and *F. oxysporum*. From the pathological diagnosis conducted, these fungal species can be considered as pathogens that affect the seeds of the evaluated legume.

On the other hand, although *P. expansum* and *A. niger* (fig. 3) produced discoloration of the seed coats, they can also cause the rotting of the germinated

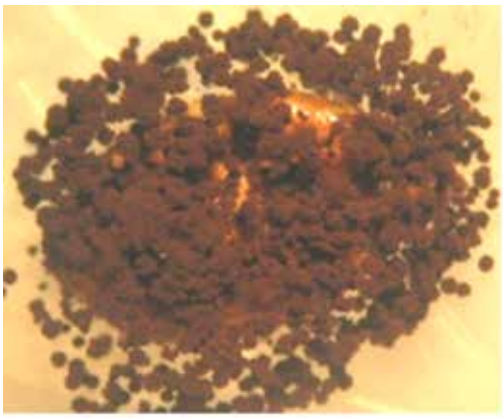

Figure 3. Rotten seed of *L. leucocephala* cv. Peru with 18 months of storage, infected by *A. niger*.

seeds and their death, for which seedling germination and emergence would be reduced. This coincides with the report by Sadhu (2014) for *A. niger*, in seeds and seedlings of *Vigna radiata* L.

In the literature related to *L. leucocephala* cv. Peru, no information has been found about the negative effects exerted by the above-mentioned fungal agents, or about the germination process. However, it is stated that during storage the *Aspergillus* and *Penicillium* species cause the accelerated deterioration of seeds, through their warming, the loss of their nutritional properties, the production of toxins, the rotting and the loss of viability (Anon, 2010).

Likewise, it is reported that the fungi that belong to these two genera represent the most common microflora in the storehouse and cause the highest infections in the seeds (Amaral and Lemos, 2009), aspect that was corroborated in the *L. leucocephala* seeds in this study

The lower incidence exerted by *F. oxysporum* in the stored seeds is characteristic of the field fungi, which show their supremacy under those conditions (high temperature and relative humidity).

CONCLUSIONS

The 11 fungal species found during the germination of *L. leucocephala* cv. Peru seeds belong to seven representative genera of the field and storehouse fungi: *Fusarium, Pestalotia, Cladosporium, Penicillium, Rhizopus, Trichoderma* and *Aspergillus.*

The fungus that showed the highest infection percentage in the seeds of both ages of storage (germinated and rotten) was *P. expansum*, which reached its maximum expression in the 18-month rotten seeds.

The main symptoms that appeared in the seeds were: discoloration (caused by *P. expansum*, *A. niger*, *Pestalotia* sp., *Trichoderma* sp., *A. flavus* isol. 2 and *F. oxysporum*), spots on the cotyledons and necrosis of the radicle (caused by *A. flavus* isol. 2 and *F. oxysporum*).

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