Multivariate analysis of the temporal changes of fungal communities in unsafe storage conditions of some common wheat varieties in relation to relative humidity level and rice weevil infestation

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Abstract

Fungal colonization of stored grain bulks is a major threat for mycotoxin contamination and reduction in viability of grain when stored under unsafe conditions, e.g. under high r.h. and insect presence. An investigation was carried out to identify the trends of the changes in the fungal species communities during storage of wheat grain under these unsafe storage conditions. The distribution change of fungi genera was monitored on small grain samples of three wheat varieties with different kernel size and hardness (soft, medium-hard and hard), during 160 d storage at constant temperature of 22-23°C, at two r.h. levels, and with or without an infestation by the rice weevil *S. oryzae*.

According to their behavioral differences related to grain water activity affinity, fungi genera were classified in three groups: i/ The hydrophilic group of field fungi (*Fusarium*, *Geniculifera*, *Sepedonium*, and *Chrysogenum*); ii/ The intermediate semi-xerophilic fungi (*Alternaria*, *Mucor*, *Ulocladium, Epicoccum,* and *Arthrobotrys*); iii/ The storage xerophilic fungi (*Penicillium* and *Aspergillus*). Temporal abundance of these three groups with grain storage time and condition was observed in weak relation with wheat variety and insect presence. The multivariate comparison of the different experimental situations revealed a difference in the susceptibility of varieties to fungal species colonization in close relationship with the final equilibrium level between ambient r.h. and grain moisture content which was observed variety-dependent. This difference was not related to grain hardness but rather to a different r.h. affinity. For one variety (*Apache*), the germination rate was declining more rapidly than for the two others with storage time. Any significant relation between sound and infested grain condition and the contamination rate by storage fungi could be found. The susceptibility of the three wheat varieties to critical storage conditions and fungal colonization may lead in one variety to a hot-spot formation.

Keywords: Common wheat, Variety, Fungal microflora, Insect pest, Fungi abundance change

1. Introduction

In France, freshly harvested wheat is never dried and is stored directly without any modification of its original physical-chemical condition at the harvest. The legal limit of moisture content recommended for safe storage of grain is fixed at 15.5% (wet basis) maximum. Nevertheless, part of the domestic production of wheat (common and durum wheat) is exported toward EU third countries located in Mediterranean or tropical climatic areas. In these warmer storage conditions, wheat has a much higher potential for quality loss by deteriorating agents, not only insect infestation but also by fungal microflora infection in relationship with the overcoming of the threshold of water activity allowing more or less xerotolerant fungi species development (Cahagnier at al., 2005). To face this quality retention storage issues that may occur in countries importing grain from developed countries, generally well equipped to prevent or reduce the grain quality deterioration process, grain stores managers are often without effective intervention means. When temperature and moisture content are high, grain respiration becomes active. The heat produced mainly by microorganisms living in the grain bulk increases the temperature of the grain that indirectly favored the fungal growth (Fleurat-Lessard, 2004). Among the various living organisms in the stored grain ecosystem, the storage fungi represent the major cause of deterioration of grain quality and of commercial value, in relationship with the potential of certain species for mycotoxin production, especially when grain is stored under a warm climate environment. This change in

temperature of stored grain can result in a spontaneous heating from the growth of fungi and in the colonization of the surface of grain bulks by thermophilic fungi and actinomycetes (Fleurat-Lessard, 2002; Magan and Aldred, 2007). In condition of medium wet or moist grain at harvest, the fungi species belonging to the group of the 'hydrophylic field flora' are predominant during the beginning of the storage period. During long-term storage, xerophilic fungi species (called the 'storage flora') progressively replace the field flora over a period of several months of storage (Pelhate, 1982; Frisvad, 1995; Fleurat-Lessard, 2002). In agreement with the concept of Wallace and Sinha (1981), the stored grain ecosystem must be considered by a holistic and ecological approach to enable a proper understanding of the processes occurring and to improve strategies of post-harvest protection against deteriorative forces (Tipples, 1995; Magan and Aldred, 2007). There is very little detailed information on the tolerance and susceptibility of wheat varieties actually cultivated in France to critical storage conditions. To improve the actual knowledge about wheat varieties sensitivity to poor storage conditions, the impact of critical conditions of storage on stored grain qualitative trait changes were investigated recently and results of this systemic study are presented in another session of the present Conference (Fourar et al., 2010). However, in the same trial, we examined more deeply and in an ecological manner the patterns of evolution of fungi species communities in comparing two opposite storage conditions, a safe environment and a critical condition of a_w and insect infestation.

Our main objective was to relate the dynamics of the changes in fungus species community to the deterioration process of wheat quality traits. The questions to be tackled were: i/ May grain hardness influence fungal species community change trends during long-term storage in critical physical-chemical conditions? ii/ The currently cultivated wheat varieties have they different susceptibilities to critical storage conditions and especially when the storage mycoflora may develop?

2. Materials and methods

2.1. Experimental design

A multidimensional laboratory trial was carried out to identify the key-factors of the overall quality traits changes, to understand their interactions in the process of deterioration, and finally to reveal underlying trends of critical storage conditions that may endanger grain quality retention. A large set of qualitative criteria was followed on grain batches from 3 wheat varieties with various qualities for cereal food processing, which were stored during 160 d at 22-23°C, under two different relative humidities (r.h.), and with or without an infestation by the rice weevil *S. oryzae*. The major factors involved in wheat grain quality trait changes in relation to the development of insect and fungi populations were periodically recorded each 40 d approximately. At each checking date, all the grain quality attributes were determined by standard methods or by laboratory proofed methods (Table 1).

Table 1 List of quality traits of wheat grain with the reference of each analytical method used for their quantification in the present study. *Grain quality traits are distributed in the different classes of quality attributes*.

Grain Quality trait analyse	acronym	Analytical method	Reference
1. Sanitary and soundness condition			
Adult insects counting	Insect_AD	Sieving – NF-V 03-742	Afnor, 1982
Insect hidden infestation counting	Insect_HI	Radiograph-ISO 6632-4	Afnor, 1982
2. Microbiological spoilage			
2.1 Qualitative analysis: Rate of fungi-contaminated kernel	Cont_Rate	Ulster's method	Cahagnier and Richard-Molard, 1997
2.2 Quantitative analysis: Isolation and identification of fungal colony-forming-unit (CFU) per g	Fungi_Q	NF V08-011	Afnor, 1996
3. Germination			
Germinative capacity	Germ_Cap	ISTA rules for seed testing	ISTA 1999
4. Physical-chemical condition			
Moisture content (wet basis)	MC	Oven-drying practical method NF V 03-707	Afnor, 1982

Grain Quality trait analyse	acronym	Analytical method	Reference
Kernel hardness	Hardness	Hardness point-meter	Hardness meter notice
Thousend grain mass	TGM	NF V 03-702	Afnor, 1982
5. Biochemical composition			
Lipid acidity (or fat acidity)	Lipid_Ac	NF V 03-712	Afnor, 1982
6. Statistical analyses			
Multivariate explanatory analyses		Multiple correlation - PCA	Addinsoft, 2005

The wheat varieties batches, that had been cultivated especially for this trial in center-northern France, were received just after 2007 harvest and were fumigated with phosphine before use. Then, a thorough mechanical and manual cleaning was achieved to sort all impurities and abnormal kernels before settling the experimental samples. The experimental design was hierarchical, composed with three levels of controlled factors: Variety, ambient r.h. and infestation with *Sitophilus oryzae* (L.) (*vs*. uninfested control). For each level of factor, four grain sample of 1.150 kg each were placed in aerated glass containers and put inside a controlled-r.h. storage enclosure. We applied the approach of the "fixed-effect-modelling" in which several qualitatively and logically distinct variables were checked on the same grain sample at different time intervals during a storage period of 160 d. This is a covariance analysis situation where several treatments (different grain varieties and storage conditions) were applied to the objects of the experiment (grain samples) to see if the response variable values changed along time. Among the observed variables, the fungi species distribution and occurrence were more particularly investigated.

2.2. Analysis of fungi community evolution during storage

During the 160-d storage period, the grain was sampled four times after 42, 75, 12, and 160 d. A part of the sample was used to determine the rate of contamination of kernels by the Ulster method (Cahagnier and Richard-Molard, 1997). Another part was used to achieve a global quantitative microbiological analysis on two replicates of each experimental unit (giving the global count of colony-forming-unit (CFU) per g of grain). The fungi species colonies observed on Petri dishes medium of the quantitative microbiological analysis were counted in separated classes according from their macroscopic external aspect. The formal identification of the genus of each separated "class" was performed after isolation on two different culture media (Potato dextrose agar (PDA), malt and yeast extract agar). The frequency of the distribution of each identified fungi genus was then calculated for each experimental condition (weighed mean percentage) and for each control date.

2.3. Statistical analysis

The multidimensional statistical analysis and chart plotting were achieved with Xlstat® (Addinsoft, Paris, France, 2007) software. In our experiment, multivariate data were expressed in a matrix form with *p* columns (measured variables including observed fungi species) and *n* rows (wheat varieties sample units distributed in various storage conditions of r.h. and insect infestation). The variables were ranged in two classes: i/ Explanatory variables: r.h. level (*r.h._Equi*), insect presence *vs*. absence (*Infested*), grain hardness (*Hardness*, related to varietal difference), and storage time (*Time*); ii/ Dependent variables: adult insect density per kg (*Insect_AD*), insect hidden infestation (*Insect_HI*), germination capacity (*Germ_Cap*), fungi CFU per g (*Fungi_Q*), grain contamination rate by fungi (*Cont_Rate*), fat acidity (*Fat_Acid*), and 11 different *Genera* of fungi recovered from wheat grain samples along the study.

The interactions between all variables were represented and analyzed in a multivariate global approach. A multivariate covariance analysis (ANCOVA) was performed in Xlstat® to assess the effect of storage conditions and of the prime characteristics of varieties (imbedded in *Hardness*) on all dependent variables, and especially on the fungi distribution variation trends. This data processing software allowed carrying out the calculation of simple and multiple correlation coefficients, as also the analysis of covariance enabling to model by multiple correlation the evolution of each dependant variable as a function of the explanatory variables. Next, data multivariate analysis results were processed using the principal component analysis (PCA). This procedure allowed extracting the Pearson's product moment correlation matrix of the binary correlations between all the dependant and explanatory variables. PCA

was performed to visualize the circular diagram of the strength of the correlations between all variables in eliminating the effects of redundancy between the variance of closely related variables (e.g. protein content and hardness). This diagram allowed to precisely appreciate their complex interactions from graphically represented independent variables taken in a whole set.

3. Results

Two main sets of results were intended: i/ descriptive results of the changes in the level of dependent variables during the 5 months storage period as also the comparison between variation trends related to safe or critical storage environment conditions; ii/ Explanatory analysis of the global interactions between storage conditions (in critical situation) and qualitative traits change with time allowing to understand the involvement of fungi species communities into the quality deteriorative process.

3.1. Analyse of variation trends in fungi distribution

A global overview of the changes in the rate of contaminated seeds of the three wheat varieties stored in the different conditions exposed above showed that all the kernels were harbouring at least a fungal germ, whatever the variety (Fig. 1). This 100% contamination level was declining during storage from 4 months storage time for *Caphorn* variety (hard wheat type), and from 3 months storage time for the two other varieties (*Apache* and *Crousty*, respectively medium-hard and soft). However, at the end of the storage period this global contamination rate was approximately the same for the three varieties (between 78 and100% fungi-contaminated kernels).

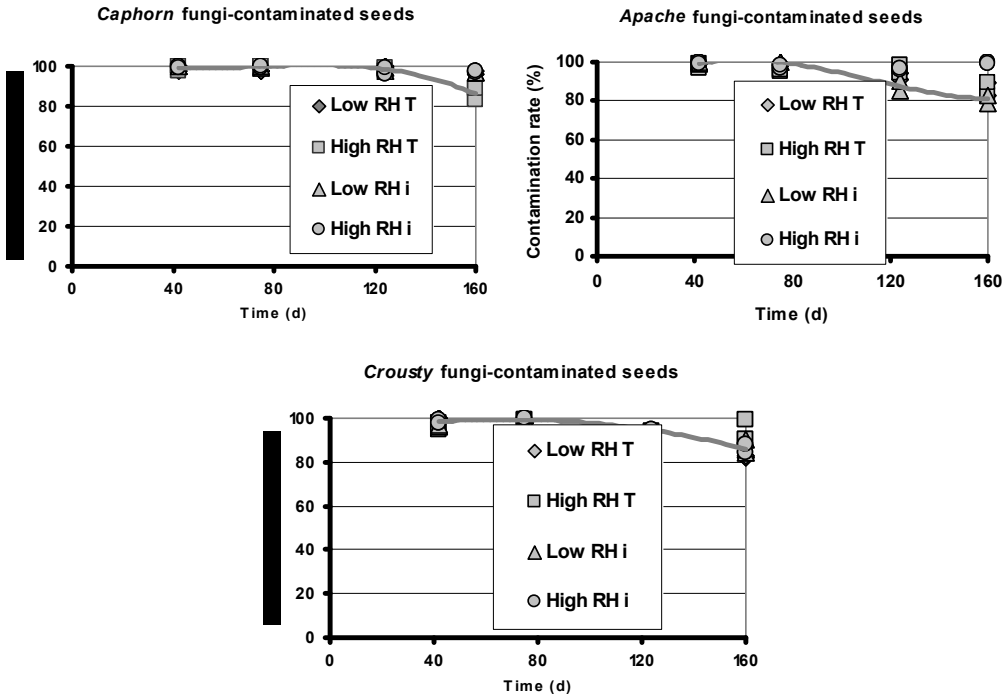

Figure 1 Changes in the rate of contaminated seeds observed on three wheat varieties stored under high or low r.h. and with and without an infestation by *S. oryzae* (T = uninfested control; i = infested).

The identification of fungi *Genera* enabled to establish a comparative distribution of fungi species in relation with storage conditions (Table 2).

Table 2 Comparative distribution of fungi genera isolated in samples of three wheat varieties during a 160-d storage period in two different r.h. levels and with and without an infestation by *S. oryzae.*

Wheat variety	**Caphorn**		**Apache**		**Crousty**	
Fungus Genus	**Control (sound)**	**Infested**	**Control (sound)**	**Infested**	**Control (sound)**	**Infested**
Fusarium	+++	+++	+	+	++	++
Epicoccum	+	+	+	+	+	+
Aspergillus	+	+	+	+	+	+
Penicillium	+	+	+	+	+	+
Alternaria	++	++	+	+	+	+
Chrysosporium	++	+	++	+	++	++
Geniculifera	+++	+++	+++	+++	+++	+++
Sepedonium	+	+	+	-	+	++
Ulocladium	++	+	++	+	++	++
Mucor	+	+	-	-	-	-
Arthrobotrys	++	++	+	++	++	+
Other *Taxa*	+	+	+	+	+	+

Among the 11 formally identified *Genera*, the most frequent fungi were: *Fusarium*, *Geniculifera*, *Chrysosporium*, *Alternaria*, *Ulocladium* and *Arthrobotris*. Most of these *Genera* corresponded to a primary contamination of grains at the harvest and they belong to "field mycoflora" or to "intermediate mycoflora" according to the classical grain fungi dynamics series concept (Sinha, 1979; Pelhate, 1982).

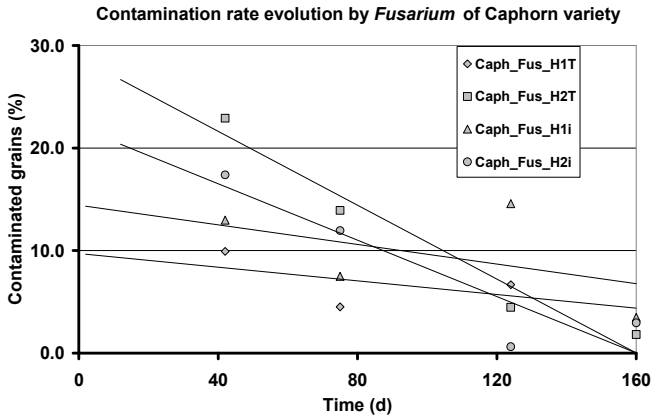

Figure 2 General trends of decrease of the contamination rate of *Caphorn* wheat variety by field fungi of *Fusarium* spp.

These two series of grain mycoflora had a different fate along the storage period. The contamination rate by "field fungi" *Genera* as *Fusarium* spp. was regularly declining during the 5-month storage period, down to complete disappearance (Fig. 3). The fate of the "intermediate mycoflora" showed a congruent figure with the concept of grain fungi dynamic series (Fig. 4). Again according to the theory, the appearance of "xerophilic storage fungi" occurred only after 4 months of storage (Fig. 5).

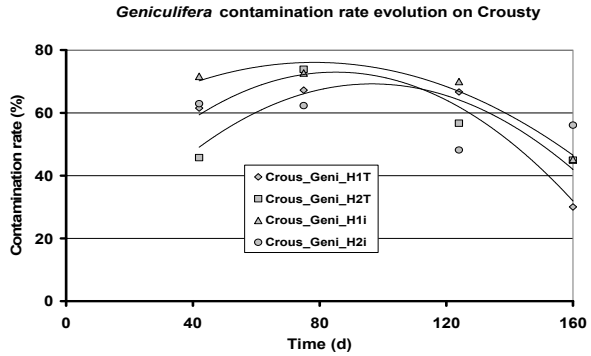

Figure 3 General trends of evolution of the contamination rate of *Crousty* wheat variety by semi-xerophilic fungi (intermediate mycoflora group) of *Geniculifera* spp.

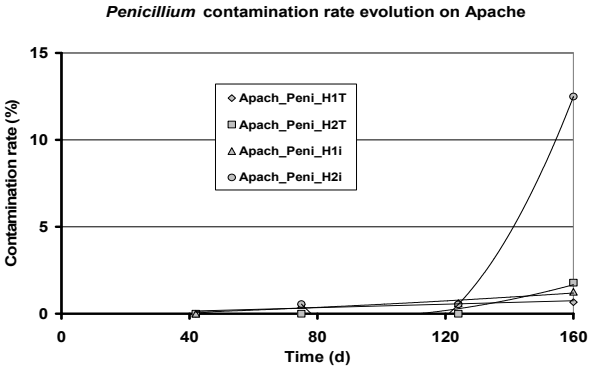

Figure 4 General trends of evolution of the contamination rate of *Apache* wheat variety by xerophilic fungi (storage mycoflora group) of *Penicillium* spp.

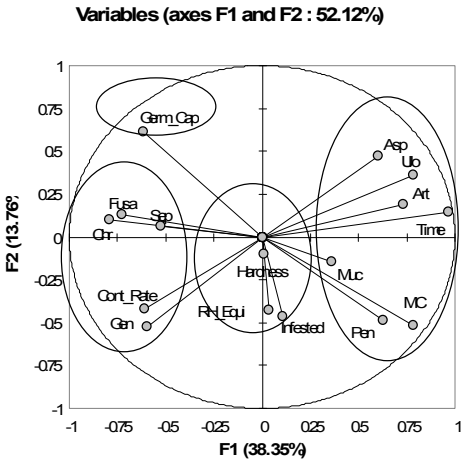

Figure 5 PCA: circular diagram visualising the correlations between all variables (dependent and explanatory) revealing the interactions in some qualitative traits and in fungal microorganism community structure during a 160-d storage period of 3 wheat varieties of different hardness at two different r.h. levels and with or without an infestation by *S. oryzae*.

3.2. Multivariate explanatory analysis of relationship between grain storage condition and fungi species distribution change

A linear multiple regression was computed for the predictive modelling of each dependent variable (including each fungi *Genus* frequency) as a polynomial function of the explanatory variables: kernel hardness (major discriminative attribute for each variety), r.h. level, presence or absence of insects, and variety specific effect. The analysis of this multiple linear regression showed that the dependent variables could be correlated with the set of explanatory variables, except total counts of fungal CFU (*Fungi_Q*) (Table 3).

Table 3 Descriptive parameters of multiple regression models predicting the dependent variables (see Table 1) as a polynomial function of explanatory variables: kernel hardness (imbedded in variety properties), r.h. level equilibrium, presence or absence of insect (*Infested* vs. control) and variety specific effect.

Explanatory variables	**# F-test value**	**Hardness**			**RH Equilibrium**			**Insect infestation**		
Dependent variables		**Value**	**t**	**Pr > [t]**	**Value**	**t**	**Pr > [t]**	**Value**	**t**	**Pr > [t]**
Moisture content	19.405***	0			0			0		
Germ_Cap	11.606***	0			0			-0,332	-2,745	0.009**
Fungi_Qu	NS									
Cont_Rate	18.18***	0			0			0		
Fusarium spp.	27.628***	0			0			0		
Aspergillus spp.	13.029***	0			-16,16	-1,332	NS	0		
Penicillium spp.	9.155***	0			6,004	1,142	NS	0		
Chrysosporium sp.	31.166***	0			0			0		
Geniculifera sp.	18.920***	0			0			0		
Sepedonium sp.	10.517***	0			11,602	1,576	NS	0		
Ulocladium sp.	48.847***	0			-15,62	-0,887	NS	0		
Mucor spp.	8.296***	0			0			0		
Arthrobotrys sp.	30.464***	0			18,24	1,787	NS	0		

Continue:

Explanatory variables	**# F-test value**	**Storage time**			***Caphorn* variety**			***Apache* variety**		
Dependent variables		**Value**	**t**	**Pr > [t]**	**Value**	**t**	**Pr > [t]**	**Value**	**t**	**Pr > [t]**
Moisture content	19.405***	0,02	6,784	< 0.001***	-0,331	-1,005	NS	0,788	2,393	0.021*
Germ_Cap	11.606***	-0,479	-3,959	< 0.001***	0					
Fungi_Qu	NS									
Cont_Rate	18.18***	-0,069	-6,11	< 0.001***	4,625			0,344	0,277	NS
Fusarium spp.	27.628***	-0,085	-7,515	< 0.001***	1,012	0,805	NS	-5,021	-3,992	< 0.001***
Aspergillus spp.	13.029***	0,066	4,928	< 0.001***	0			0		
Penicillium spp.	9.155***	0,024	4,124	< 0.001***	0			0		
Chrysosporium sp.	31.166***	-0,059	-9,014	< 0.001***	-0,844	-1,167	NS	-2,489	-3,441	0.001***
Geniculifera sp.	18.920***	-0,204	-6,553	< 0.001***	-2,69	-0,781	NS	9,504	2,758	0.008**
Sepedonium sp.	10.517***	-0,035	-4,307	< 0.001***	0			0		
Ulocladium sp.	48.847***	0,192	9,844	< 0.001***	0			0		
Mucor spp.	8.296***	0,012	3,144	0.003**	1,357	3,241	0.002**	-0,091	-0,216	NS
Arthrobotrys sp.	30.464***	0,086	7,6	< 0.001***	0			0		

The best fitted polynomial regressions were found with the following fungi *Genera*: *Ulocladium, Chrysosporium, Arthrobotrys, Fusarium, Geniculifera*, and *Aspergillus*. However, the explanatory variables did not explain the same amount of variance for each dependent variable. Thus, storage time (*Time*) was observed a very highly significant component of the predictive model of all dependent variables, except *Fungi_Q* (Table 3). Infested condition (*vs*. sound grain condition) was negatively and

highly significantly correlated with germination capacity and without relation with any fungi *Genus* variance. The contribution of the variety Apache to the variance of moisture content was significant. This positive correlation indicated that the higher increase in moisture content in *Apache* than in the two other varieties might be related to a higher r.h. affinity of this variety (Fourar-Belaifa et al., 2010). For the contribution of the varieties to the variance of fungi *Genus*, it was observed two negative correlations between *Apache* variety with *Fusarium* and *Chrysosporium Genera*, and a positive correlation with *Geniculifera Genus*. *Caphorn* variety had a significant positive correlation with *Mucor* Genus only.

The graphical representation of the magnitude of correlation between variables by PCA circular diagram allowed extracting the more relevant interactions between variables that are significantly involved in the deterioration process during storage in critical conditions (Fig. 5). With PCA graphical representation, it was observed that the first component axis contributed to more than 38% of total variance or overall correlations of the whole set of variables. The fungi *Genera* were distributed into two distinct groups at each end of this first component. The first group included *Genera* negatively correlated with storage time and moisture content variables. This meant that these fungi *Genera* regressed with storage time and low grain moisture content (Table 4). Thus, these Genera could be clearly classified as 'hygrophilic field fungi' type: *Fusarium*, *Sepedonium*, *Chrysosporium*, *Geniculifera*. At the opposite end of the first axis, the second group of fungi *Genera* was correlated to storage time in significant dependence with moisture content variation. This meant that the dynamics of these fungi *Genera* was dependent of long-term storage periods and that their occurrence was dependent of moisture content variance. Thus, these *Genera* might be classified as 'storage or intermediate mycoflora': *Aspergillus*, *Ulocladium*, *Penicillium*, *Arthrobotrys*, and *Mucor*. The proximity between explanatory variables "*Infested*" and "*r.h._Equi*" and dependent variables *Penicillium* and *Mucor* Genera indicated that the presence of insect and high r.h. might have induced an abundant proliferation of storage fungi, which was a situation observed with *Apache* variety at the end of the storage period (hot-spot induced by storage fungi of *Penicillium* Genus, Fig. 4). Neither hardness, nor r.h. level had a significant contribution to the overall correlation of the whole set of variables processed through PCA. The second axis of PCA contributed weakly to the overall covariance of the whole set of variables (less than 14%). Along this second axis, it was observed a negative influence of insect infestation on germination capacity. However, the influence of moisture content level on germination capacity was significantly correlated (correlation coefficient: -0.842; d.f.: 5, 42; $P \leq 0.05$).

Table 4 Direction of significant correlations between controlled factors and fungi species communities variance monitored on 3 wheat varieties stored during 160 d in safe or critical environmental conditions (r.h., insect infestation and kernel hardness according to selected wheat varieties)

	Time	**Infested**	**Var_Apache**	**Var_Caphorn**
germ_Cap	negative	negative		
Fungi_Qu	NS			
Cont_Rate	negative			positive
M.C.	positive		positive	
Fusarium	negative		negative	
Apergillus	positive			
Penicillium	positive			
Chrysosporium	negative		negative	
Geniculifera	negative		positive	
Sepedonium	negative			
Ulocladium	positive			
Mucor	positive			positive
Arthrobotrys	positive			

4. Discussion

Some correlations could be explained by the properties of the selected varieties. Thus, the fungi-contamination-rate of wheat kernels correlated with the hardness of kernels because the most contaminated variety at the harvest by field fungi was *Caphorn*, which was of the hard type (*vs*. medium-hard and soft for *Apache* and *Crousty*, respectively). Nevertheless, this situation could be also related to a

low susceptibility of *Apache* to the contamination by *Fusarium* spp., which was the most abundant fungi *Genus* found on *Caphorn* at the harvest. The least susceptibility to *Fusarium* contamination of Apache than the two other varieties was in agreement with the rating for intrinsic susceptibility of *Fusarium* spp. contamination published for all wheat varieties cultivated in France. An important result obtained in the present study was to see that the hardness type of wheat variety has no significant relationship with fungi *Genera* variations during post-harvest storage. Nevertheless, wheat variety intrinsic characteristics and properties had a positive or a negative dependence with several variables linked to the suitability for long-term storage without deterioration of quality traits (Table 4). From the results of this specific study investigating about the aptitude for safe storage time in a small set of three different wheat varieties currently cultivated in France, it could be deduced that the aptitude of high-yield productive wheat varieties to tolerate critical storage environmental conditions (high r.h., insect infestation and fungal spoilage risks) was not taken into account as an important quality attribute in variety creation.

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