OXYGEN DEPLETION AS A METHOD IN GRAIN STORAGE : MICROBIOLOGICAL BASIS

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1. INTRODUCTION

When compared with the bacteria of the same substrata - and namely grains- the fungi are named aerobes.Now this quality has constituted for nearly a century the biological basis of controlled atmosphere storage (Blanc, 1938 ; Oxley and Hyde, 1957 ; Poisson, 1969 ; Hyde, 1962 ... 1974 ; Masson, 1969). It is true that bacteria greatly influence microbial series in air-tight silos or depleted in oxygen ; yet more or less abundant yeasts generally accompany them and, sometimes also, some filamentous fungi. Therefore one must consider some fungi as facultative aerobes or micro-aerophiles (Bartnicki-Garcia and Nickerson, 1962, Gunner and Alexander, 1964 ; Stotzky and Goos, 1965 ; Trisvyatskii, 1966 ; Tabak and Cook, 1968a ; Curtis, 1969)...

The principle of air-tight storage has a biological basis : in an hermetic container,the metabolic processes of the very seed and the activity of the associated microorganisms reduce oxygen content in the end and accumulate carbon dioxide.Progressively the result of this is a condition first biostatic then inhibiting which could be lethal for spoilage agents and fungi above all (Milner and Geddes, 1945 ; Peterson et al. 1956)... It is then to determine the primary cause of the limiting action : anoxy or carbon dioxide supply ? Many authors have brought answers with shades of meaning in accordance with the diversity of the substrata and their associated flora (Brown, 1922 ; Golding, 1945 ; Geddes and al. 1955 ; Peterson and al. 1956 ; Follstad, 1966 ; Tuite and al. 1967 ; Escoula and Le Bars, 1973 ; Mitchell and Mitchell, 1973 ; Pelhate, 1975, 1976)...

The present work sets forth a few complementary aspects of our own investigations led from the laboratory test to the farm silo by associating the microorganisms behaviour as well as the change of the spontaneously contamined grain bulk.

2. ANAEROBIC GROWTH OF GRAIN FUNGI IN VITRO

2.1 Materials and methods

Some species said to be micro-aerophilic on silages (Pelhate, 1975, 1976, 1977) and classified in 3 ecological categories identified on grains (Pelhate, 1968a, 1979) have been submitted to culture in comparison with more cosmopolitan agents. Inoculated Petri dishes with malt agar media are set in an gas-tight container at 20 and 32°C for 10 days. Anoxy is carried out in 3 conditions :

TABLE 2.1

Growth rates of grain fungi in controlled atmospheres

(% of control in aerobic condition : mycelial growth of filamentous species, budding cells of yeasts)

Species	Atmosphere	Vacuum		CO_2 10%		CO_2 100%	
	Temperature (°C)	20	32	20	32	20	32
FİELD FUNGİ							
Alternaria tenuissima (NEES ex FR.)WİLT.		0	0	0	0	0	0
Fusarium culmorum (W.G.SMİTH)SACC.		0	0	ε	1,2	0	ε
F. poae (PECK)WOLL.		ε	1,6	6,2	7,5	ε	ε
F. sacchari (BUTL.)W.GAMS var. *subglutinans* (WOLL.et REINK.)NİRENB.		ε	ε	3,6	4,2	ε	ε
İNTERMEDİATE FUNGİ							
Byssochlamys nivea WESTL.		0	ε	12,5	16,3	7,5	9,5
Cladosporium cladosporioides (FR.)DE VRİES		0	0	0	0	0	0
Geotrichum candidum LİNK		0	0	ε	2,5	ε	1,5
Monascus purpureus WENT		0	0	ε	1,2	ε	ε
Mucor circinelloides VAN TİEGH. f. *griseo-cyanus* SCHIPPER		ε	ε	6,5	12,6	8,5	3,3
M. racemosus FR.		0	ε	1,3	4,2	ε	ε
Paecilomyces variotii BAİNİER		0	0	2,2	5,3	1,6	4,3
Trichoderma koningii Oud.		0	0	0	ε	0	ε
Candida krusei (CAST.) BERKH.		55	40	70	80	25	45
Hansenula anomala (HANSEN) H.and P.SYDOW		95	90	100	120	75	80
STORAGE FUNGİ							
Aspergillus candidus LİNK		0	0	0	ε	0	0
A. fumigatus FRES.		0	0	0	ε	0	ε
Eurotium herbariorum MALL.and CAİN		0	0	0	ε	0	0
Penicillium cyclopium WEST.		0	0	0	ε	0	0
P. roqueforti THOM		0	0	1,6	1,5	0	ε
P. stoloniferum THOM		0	0	0	0	0	0

-deep vacuum maintained by daily pumping (for a minimal instantaneous pressure of 1 mm mercury)

-carbon dioxide content of 10 % (vol.) in adequate jars ("Gaspak")

-carbon dioxide content of 100 % (vol.) obtained after deep vacuum.

Anoxy tolerance is tested through the radial growth of filamentous fungi or the cell count of yeasts (Pelhate, 1978a).

2.2 Results and discussion

The results set forth in table 2.1 show the high fungi inhibition (yeasts excepted) by anoxy. Thus some species grow in no test condition ; they must be considered as obligatory aerobes in comparison with facultative anaerobic yeasts (Tabak and Cooke, 1968a ; Trisvyatskii, 1966). Others poorly grow in deep vacuum (*F. poae* and *F. sacchari*, *M. circinelloides*) and, sometimes,only at higher temperature (*B. nivea* and *M. racemosus*) ; we consider them as micro-aerophiles (Pelhate, 1975, 1978a).

Besides the carbon dioxide incidence in the anoxic condition brings a precision to this classification of species according to two apparently contradictory effects : carbon dioxide enhances the inhibition at the higher content (*F. poae*) or it decreases it at the lower one (*Fusarium* spp., *G. candidum*, *M. racemosus* and *P. roqueforti*) and sometimes even at the higher rate (*B. nivea*, *M. circinelloides* and *P. variotii*). These data are in accordance with previous observations relative to mycoflora of diverse food-stuffs(Bottomley and al. 1950 ; Burmeister and al. 1966 ; Escoula and Le Bars, 1973 ; Mitchell and Mitchell, 1973 ; Watt, 1973 ; Pelhate, 1975, 1976) or to isolated species : *B. nivea* (Yates and al. 1967), *F. sacchari* = *F. moniliforme* (Tuite, 1961), *G. candidum* (Apelbaum and Barkai-Golan, 1977, *Mucor* sp. (Tuite and al. 1967), *T. koningii* (Walsh and Stewart, 1971) and various species (Golding, 1945 ; Milner and Golding, 1949 ; Durbin, 1955 ; Watt, 1973).

The eventual disparities in estimating specific tolerances can be explained by inevitable technical imperfections (vacuum degrees, gas purity...) or the interplay of manifold factors such as temperature, nature and weight of the substratum, complexity of the floristic series and the resulting specific interactions. The temperature acts directly on growth but also indirectly by diminishing carbon dioxide solubility in the substratum and consequently the inhibiting ability of the latter (Golding, 1945 ; Tuite and al. 1967).

The interplay of the ecological factors will be talked in the following chapter. In summation it appears that fungi are more sensitive to oxygen depletion than to carbon dioxide excess ; and when some species can grow well in anoxy, it is admitted that they utilize the most imperceptible oxygen traces included in the substratum (micro-aerophilic species) or that they modify their metabolism (facultative anaerobes those yeasts) through fermentary processes instead of respiratory ones namely for carbon dioxide (Bartnicki-Garcia and Nickerson, 1962 ; Parr and Smith, 1970) or nitrogen (Tabak and Cook, 1968b). This adaptation may entail original morphosis (Bartnicki-Garcia and Nickerson, 1962 ; Iralu, 1971 ; Escoula and Le Bars, 1973) or sometimes mutant types (Storck and Morill, 1971).

3. PRESERVATION TEST OF WET SOYBEAN IN CONTROLLED ATMOSPHERES

3.1 Materials and methods

The very wet grains (moisture content of 30.5 % w.w) are distributed in hermetic flasks : these communicate with a vacuum apparatus and gas containers with carbon dioxide or nitrogen). The atmospheres differ from one another by their composition : confined air, partial vacuum, nitrogen, carbon dioxide and mixtures of these gases ; incubation temperatures were 5, 20 and 30°C. The behaviour of the grain was studied in reference to biological parameters : dynamics of the flora, viability of seeds, or technical ones : soluble proteins, acid number (Thériault, 1978 ; Pelhate and Thériault, 1979).

The present work retains only the first aspect. The microflora spontaneously associated to grains is followed in two ways : on one side, germ counts for bacteria and yeasts superficially grown, on the other, mycelial detection on or in kernels for filamentous fungi. In the first case the germs are suspended by simply stearing grains in sterile solution. The second method is justified by the fact that many species no longer sporulate in controlled atmospheres (Brown, 1922 ; Bartnicki-Garcia and Nickerson, 1962 ; Burmeister and al. 1966 ; Iralu, 1971) ; superficial desinfection of grains before their plating essentially allows detection of internal flora in comparison with total flora.

All these methods have been previously described (Pelhate, 1968a,1970, 1979).

3.2 Results and discussion

In every test condition growth of bacteria and yeasts is obviously fore-most (See Fig.3.1) while the growth of molds in more or less limited (See Fig. 3.2)

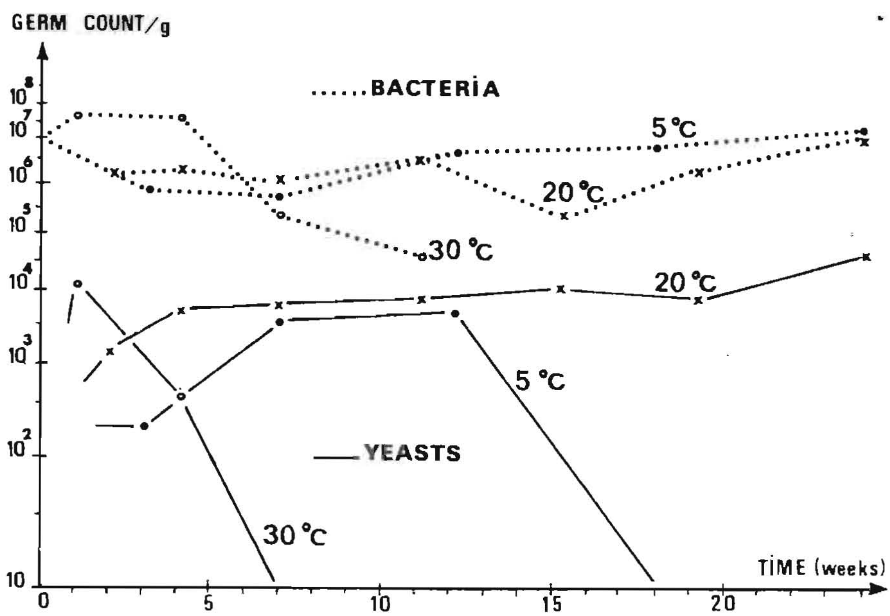

Fig. 3.1. Bacteria and yeasts dynamics on soybean stored in air-tight flasks at 5, 20 and 30°C.

in accordance with the observations of chapter 2. At determined temperature, selective inhibition is more or less identical in spite of the initial diversified test condition (atmosphere composition) ; that can be explained by high grain moisture which triggers off active metabolism of grain-flora complex so that resulting anoxy and carbon dioxide accumulation (See Fig. 3.3) rapidly make ecological environment uniform (See Fig. 3.4).

Hence temperature seems the most prevalent factor in determining of grain behaviour and above all of flora dynamics. This thermal effect is shown according to two trends : on one side, increase of all biological processes (along with

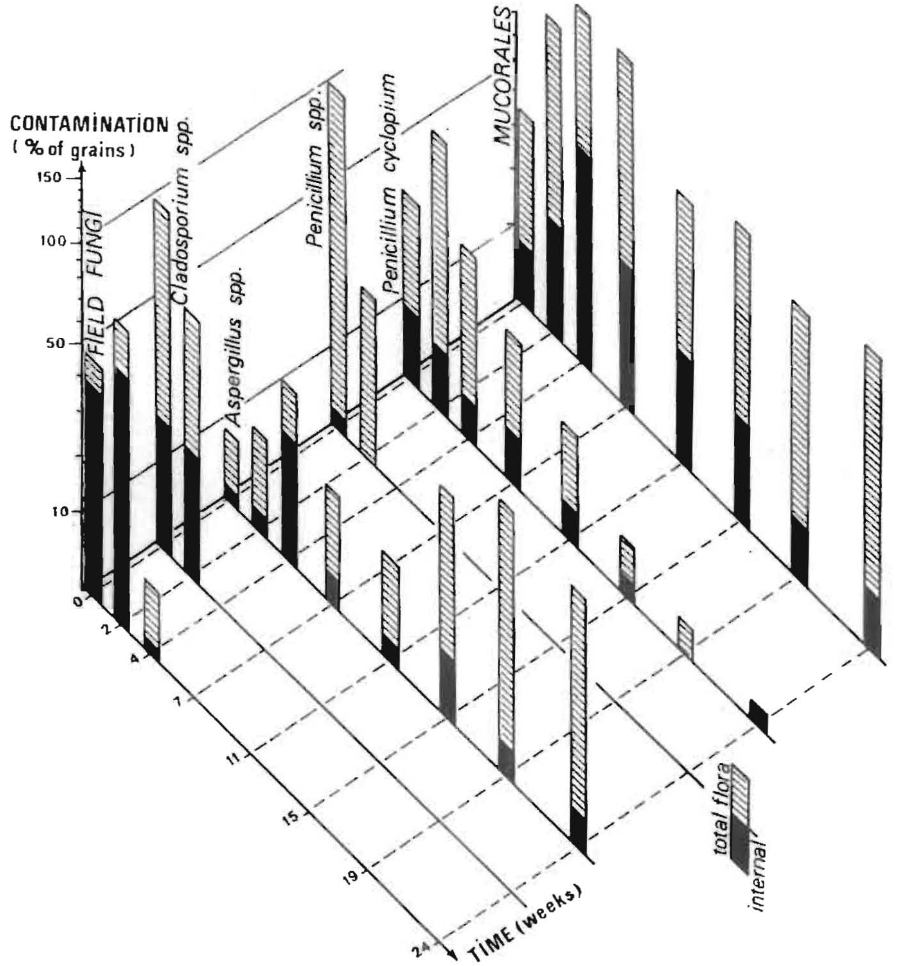

Fig. 3.2. Selective inhibition and dynamics of molds of soybean stored in confined air at 20°C.

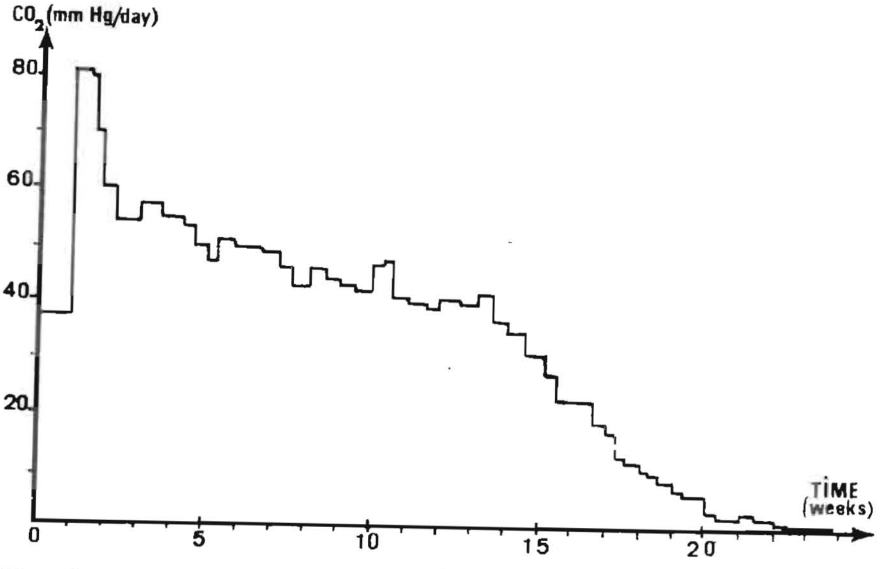

Fig. 3.3. Carbon dioxide production from soybean stored in partial vacuum at 20°C.

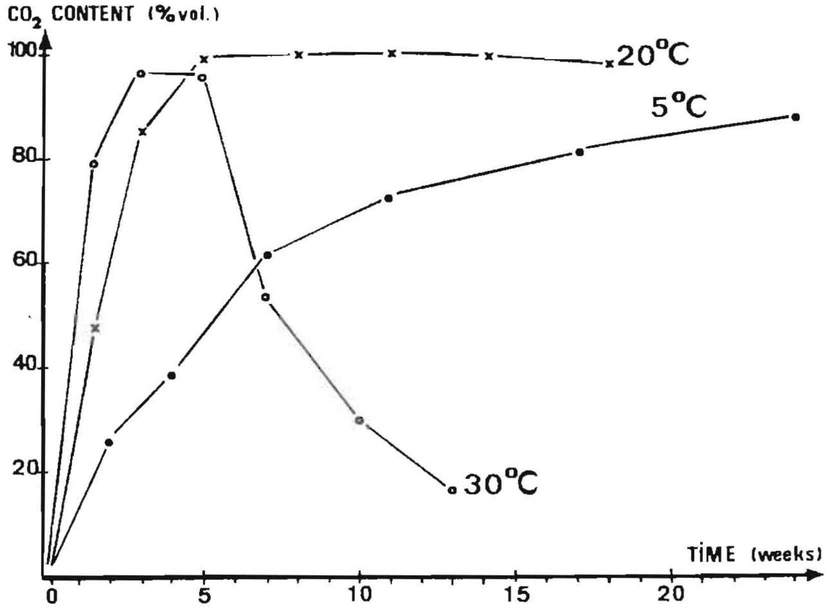

Fig. 3.4. Changes in carbon dioxide content of confined air at 3 temperatures.

respiration of seeds, microorganisms growth) and on the other, determinism of floristic dynamics to the benefit of the thermotolerant and competitive species. Figure 3.5 evinces this succession controlled by the interplay of various ecological factors and particularly temperature :

- at 5°C,all processes are slowed down and namely inhibition of strictly aerobes such as A. *tenuissima* ; limited growth of bacteria and yeasts - the usual characteristics - allows the selective growth of *Penicillium* spp. and Mucorales (namely *M. hiemalis*) which will coexist. At the end of storage,Mucorales tolerate better an excess of carbon dioxide like *Aspergillus* spp. which survive as traces without growth.

- at 20°C, the decrease of aerobic species is accelerated ; competition between 3 entities, i.e., in addition to bacteria and yeasts, *Aspergillus* spp., *Penicillium* spp. and Mucorales ends to the benefit of *Aspergillus* spp. (the most tolerant species to carbon dioxide)

- at 30°C, the advantage is obviously taken to *Aspergillus* spp. (among which A. *repens* = *Eurotium herbariorum*) and Mucorales (with M. *circinelloides* f. *griseocyanus*) because of their tolerance respectively to high temperature and carbon dioxide excess.

We can conclude by refering to the complexity of microorganism population dynamics and that of determinism of the spoiling of spontaneously polluted grains stored in air-tight conditions (Carter and Young, 1945 ;Bottomley and al. 1955 ; Tuite and al. 1967 ; Clarke and Hill, 1971). Yet selective inhibition and resultant simplification of microorganism series in conformity with their differential tolerance to oxygen depletion or carbon dioxide excess suggest a promising way of research.Grains always reveal from the harvest a noticeable pollution

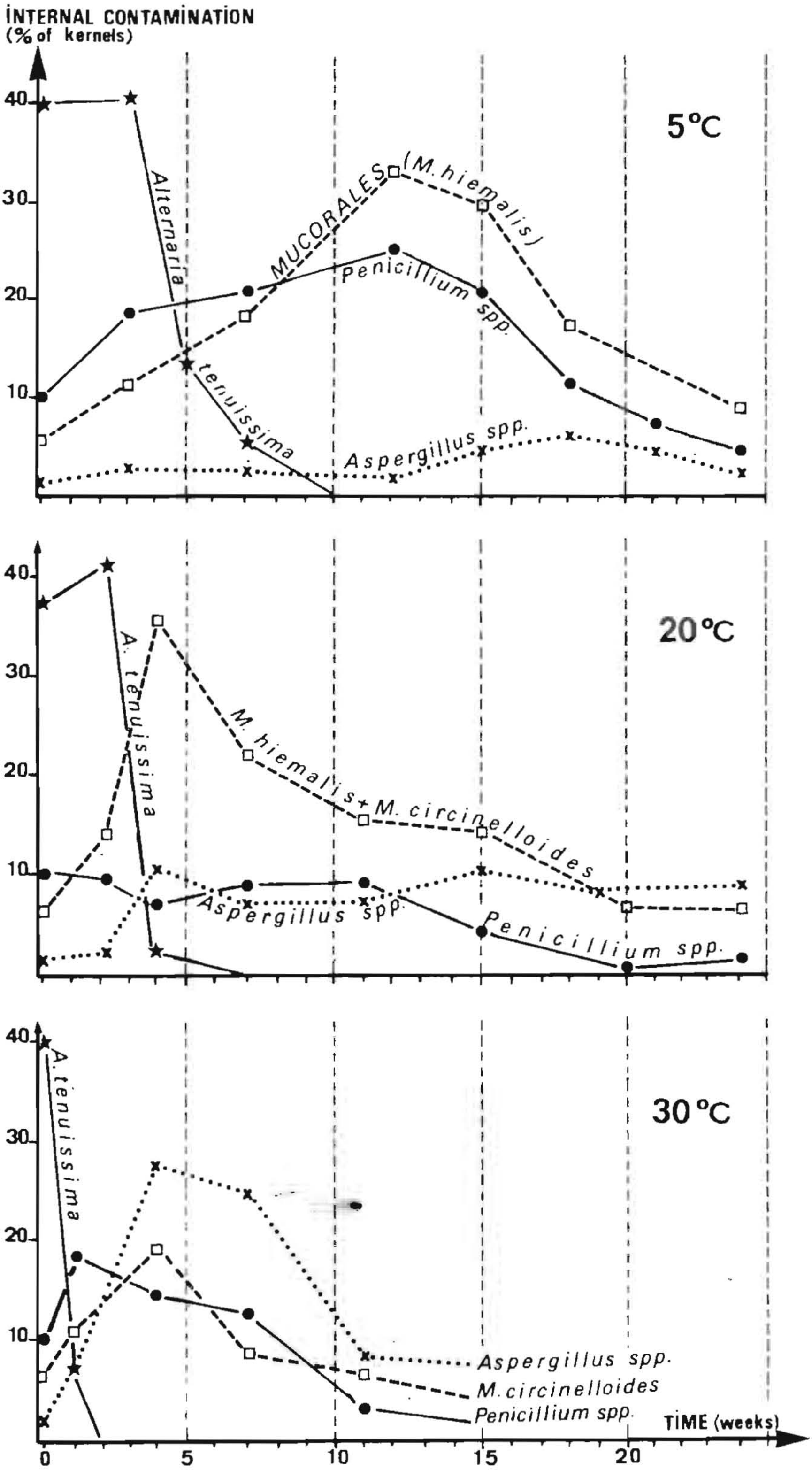

Fig. 3.5. Specific contamination of soybean in air-tight storage at 5, 20 and 30°C.

caused by various agents classified into distinct ecological types (Pelhate, 1968a, 1979) which have an adverse effect or none according to storage conditions (Pelhate, 1967, 1968b). Now wet grains are particularly susceptible to microorganisms and require adequate techniques.

Thus, in the controlled atmosphere methods some arrangements are required : improvement of structures (Mayo and Mc Neal, 1974), decrease of moisture content (Hyde, 1962...) and relatively low temperature maintained to limit biological activities. But in this case, will spontaneous anaerobiosis with its double aspect of oxygen depletion and carbon dioxide accumulation remain efficient enough to avoid the food-stuff decay ? Reducing initial intergranular air and consequently oxygen in the silo is recommended (Glass and al. 1959 ; Tabak and Cooke, 1968b ; Shejbal and al. 1973) ; the silage of wet or rehumidified crushed grain, technique more accessible to the pratician seems valid, at least in an autoconsumption system (Pelhate, 1976, 1978b).

4. PRACTICAL METHOD OF WET CRUSHED GRAIN SILAGE

4.1 Materials and methods

Two types of grains are suited to the silage method : corn (maize) with always a high moisture content at harvest time (up to 35 % w.w. and more), wheat too wet when harvested for safety condition (content moisture more than 15.5 % w.w). To avoid a complementary drying process it is now suggested to realize silage of grain as fodder but after crushing them and if needs be rehumidifying them.

4.2 Results and discussion

4.2.1 Corn

Silage behaviour is dependent on the floristic succession set forth on Fig. 4.1. In peripheral parts of the bulk unavoidable air traces allow abundant growth of mixed specied and consequently a quick and advanced deterioration. In the bulk itself, it is true, high moisture entails an early invasion of anaerobic bacteria (Lactobacilli) ; this heating and acidification stage is followed by that of yeasts. Bacteria are only in competition with hygrophilic and carbon dioxide tolerant fungi (*Aspergillus fumigatus* and Mucorales). Sometimes thermotolerant *Monascus purpureus* is in competition with yeasts after the heating stage. At the end of the process, *Byssochlamys nivea* whose performances are remarkable (See chapter 2) can be associated to Mucorales ; however fact to a weak aeration caused by opening the silo for consumption *Penicillium roqueforti*, a very common species, generally appears and masters the whole flora ; *Trichoderma* spp. and *Paecilomyces variotii* less common, rarely appear at the final stage.

4.2.2 Wheat

First attempts of wheat silage along the same line were failures as Fig. 4.2

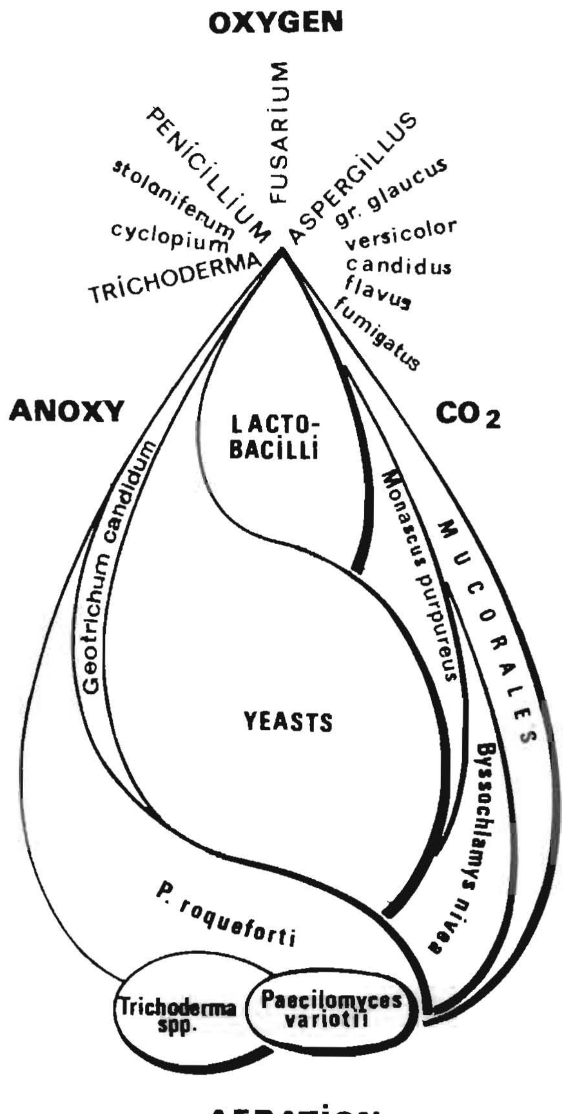

Fig. 4.1. Diagram of microflora dynamics on wet crushed corn silage (35% w.w. average moisture content).

reveals. Three stages can be distinguished in the floristic succession on damp grain principally regulated by specific needs of water (Pelhate, 1968c, 1978c) and water activity of the substratum (See Fig. 4.3).

- In the first stage, xerophilic and primary species as *Aspergillus* (= *Eurotium*) *glaucus* group can grow.
- In the second, according to progressive moisture and temperature gradients, several species actively grow and are in competition with yeasts which are limited to traces. These may be *Aspergillus versicolor* or *A. candidus* then *Penicillium cyclopium*, rarely *P. stoloniferum*.
- In the third, micro-aerophilic and CO_2 tolerant species can grow as among which rarely *A. fumigatus*, sometimes *B. nivea*, typically *P. roqueforti* accompa-

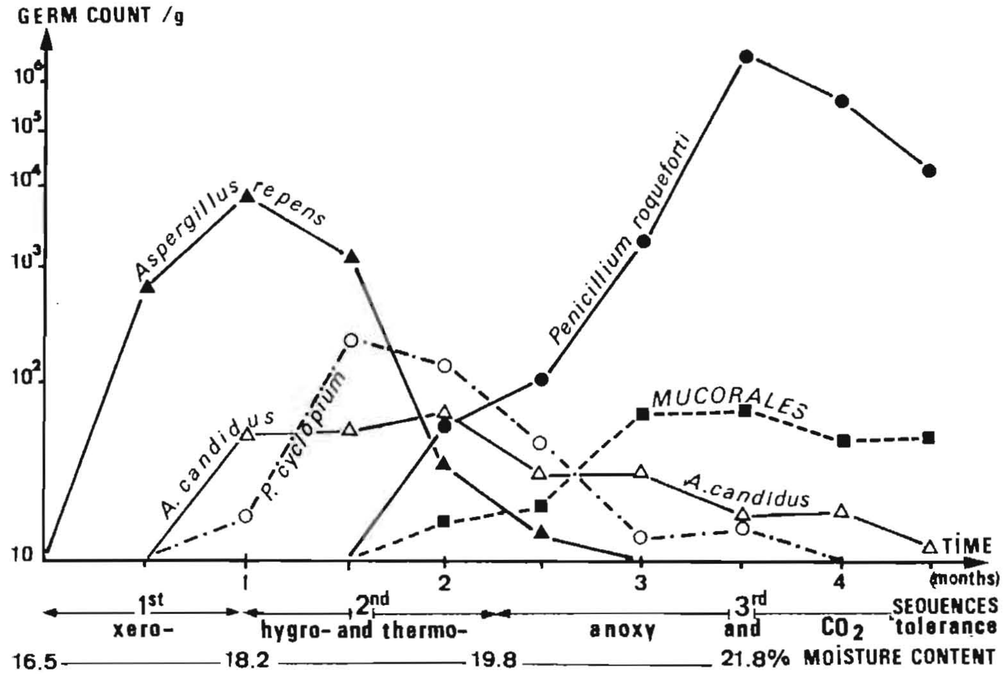

Fig. 4.2. Mold dynamics on damp crushed wheat silage (initial moisture content : 16.5 % w.w.)

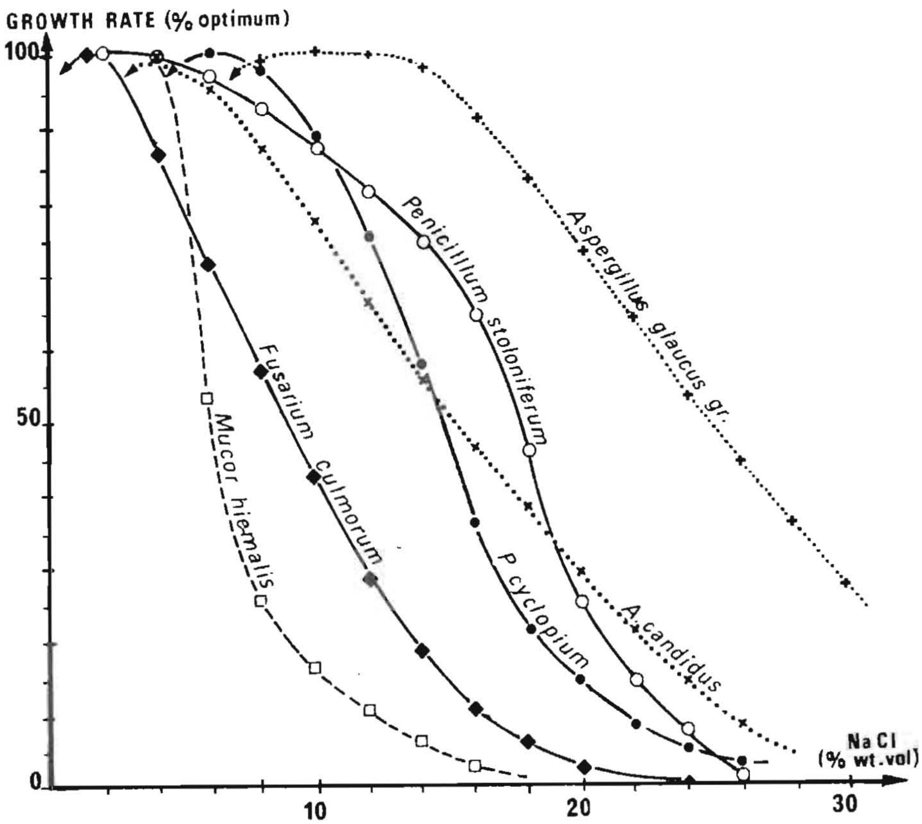

Fig. 4.3. Comparative mold growths in xerophilic condition. Water activity gradient is regulated by NaCl addition to malt agar media.

nied with Mucorales (*Mucor hiemalis*, *M. racemosus*, *M. circinelloides*).

It is therefore important for the two first stages to be avoided so that yeasts and bacteria may develop enough to inhibit spoiler molds . To that beneficial effect we recommend to rehumidify the grain up to 25 % w.w. at least at the time of crushing. It is also advised to store freshly harvested grain devoid of xerophilic storage fungi (Tuite and Christensen, 1957 ; Qasem and Christensen, 1958 ; Pelhate, 1968d). Besides it is important to improve air-tightness of structure to avoid peripheral decay (Nichols and Leaver, 1966).

The dominance of facultative anaerobic yeasts will then ensure a sufficient quality of the food-stuff meant for livestock. It has been established that wet or rehumidified grain along those lines has a better biological value (Mordenti and Zaghini, 1977).

Indeed one must not deny the fact that the food-stuff fresh from the silo had little stability (Mayo and Mc Neal, 1974 ; O'leary, 1978) ; but we say again that the method is devised on farm scale and in autoconsumption system.

5. CONCLUSION

In spite of the promising of anaerobiosis principle, practical realizations remain subject to technical difficulties such as more expensive structures and to biological incertainties i.e. pollution hazard, complex dynamics of spoiler microflora.

Yet the method brings actual advantages inherent in changes in biological equilibrium and metabolic processes ; for instance some moulds growing in even partial anoxy environment become unable to produce toxins (Mosely and al. 1971 ; Shih and Marth, 1973 ; Lillehoj and al. 1972 ; Wilson and Jay, 1975). Again air-tight storage of wet grain safeguards technological and biological quality without addition of pesticides and avoids risks of chemical toxicity.

Besides, insects often responsible for primary decay are easily eliminated (Oxley and Wickenden, 1963); in that line, the air-tight storage is justified even for dry grains.

Doubtless the method would have a greater impact if the aims are more clearly defined beforehand namely storage duration, grain utilization.

Last but no least, strictly economic considerations may determine the choice of two alternative methods i.e. complementary drying up or silage in the presence of damp grain.

SUMMARY

The oxygen content of the intergranular spaces in bulk grain has a considerable effect on the state of the microorganisms since the great majority of these are aerobes (except yeasts, a few moulds and bacterial varieties).

First in vitro experiment with controlled atmosphere, in sealed containers, pointed out that progressive anoxy is most operative condition whereas carbon dioxide in various contents can reduce or enhance the suppressive effect of

oxygen deficiency.

Second assays were carried out with soybean stored in various controlled atmospheres (air-tight condition, partial vacuum or supplied carbon dioxide...) as suitable methods for an oxygen-free condition. Specific and selective action of these modified atmospheres resulted in original and restricted series among the spontaneous microflora ; dynamics of these are obviously dependent on other ecological factors.

According to such a complementary pattern, a practical method of grain storage is suggested as the easiest and cheapest on the farm scale : damp corn or rehumidified wheat grains are crushed before storing into compressed batches.

Key words : *anaerobiosis ; anoxy ; carbon dioxide ; controlled atmospheres ; grain storage fungi ; moldy grain ; wet grain storage.*

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