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Original article

Degradation of maize stem by two rumen fungal species, *Piromyces communis* and *Caecomyces communis*, in pure cultures or in association with cellulolytic bacteria

V Roger [1, 2], E Grenet [3], J Jamot [3], A Bernalier[1]*, G Fonty [1, 4]**, P Gouet [1]

[1] *INRA - Theix, Laboratoire de Microbiologie, 63122 Saint-Genès-Champanelle;*
[2] *UFR d'Odontologie, Unité Sciences Biologiques, Département de Recherches, 63000 Clermont-Ferrand;*
[3] *INRA - Theix, Station de Recherches sur la Nutrition des Herbivores, Unité Ingestion, 63122 Saint-Genès-Champanelle;*
[4] *Université Blaise Pascal, Laboratoire de Biologie Comparée des Protistes, URA CNRS 138, Clermont II, 63170 Aubière, France*

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Summary — Two species of rumen fungi, *Piromyces (Piromonas) communis* FL and *Caecomyces (Sphaeromonas) communis* FG10, were cultured alone or in association with the cellulolytic bacteria *Ruminococcus flavefaciens* or *Fibrobacter succinogenes* on maize stem. A kinetic study of the degradation of the substrate was then made. After 48 h of culture, all non-lignified tissues observed by scanning electron microscopy disappeared with *P communis* and degradation was as complete as that observed in the rumen. In contrast, *C communis* degraded little of the plant cell walls. The ability of *P communis* to more rapidly degrade maize stem was probably due to the presence of filamentous rhizoids. The extent of dry matter loss after 8 days of incubation was practically the same in all the monocultures and in the 4 cocultures. However, the rate of degradation was faster in the bacterial than in the fungal monocultures and the co-cultures. No metabolic interaction was observed.

rumen / anaerobic fungi / cellulolytic bacteria / microbial interaction / maize stem degradation

Résumé — Dégradation de la tige de maïs par les champignons anaérobies du rumen, *Piromyces communis* et *Caecomyces communis*, seuls ou en association avec une espèce bactérienne cellulolytique. *La dégradation de la tige de maïs a été étudiée en cinétique dans des cultures pures de 2 espèces de champignons anaérobies du rumen, et dans des cocultures associant une espèce fongique à une espèce bactérienne cellulolytique* Fibrobacter succinogenes *ou* Ruminococcus flavefaciens. *Après 48 h de culture, tous les tissus non lignifiés, observés en microscopie*

* Present address: INRA, Laboratoire de Nutrition et de Sécurité Alimentaire, 78352 Jouy-en-Josas Cedex, France
** Correspondence and reprints

électronique à balayage, ont été dégradés par Piromyces (Piromonas) communis *et la dégradation est aussi complète que celle observée* in vivo. *Par contre,* Caecomyces (Sphaeromonas) communis *s'est montré moins efficace dans la dégradation des parois végétales. La plus grande efficacité de* P communis *peut être attribuée à la présence de rhizoïdes. La quantité de matière sèche disparue après 8 jours de culture a été pratiquement la même dans toutes les monocultures et les 4 cocultures. La dégradation a cependant été plus rapide dans les monocultures bactériennes que dans les monocultures fongiques et dans les cocultures. Aucune interaction métabolique n'a été observée.*

rumen / champignon anaérobie / bactérie cellulolytique / interaction microbienne / dégradation de la tige de maïs

INTRODUCTION

Anaerobic rumen fungi have the ability to hydrolyze all polysaccharides except pectin in the plant cell wall, producing formate, acetate, lactate, ethanol, carbon dioxide and hydrogen (Mountfort, 1987; Orpin and Joblin, 1988; Fonty and Joblin, 1990). All the fungal species produce an extremely wide range of polysaccharidases and glycosidases (Fonty and Joblin, 1990). *In vitro*, they are able to degrade large amounts of incubated plant material (Akin *et al,* 1983). Theodorou *et al* (1989) showed that a strain of *Neocallimastix* removed about 53% of the cell walls or about 75% of the structural polysaccharides in Italian ryegrass hay after 6 d of incubation. However, species vary in their abilities (Gordon and Ashes, 1984; Orpin, 1983, 1984; Bernalier *et al,* 1991) and little is known about the specific role of each species in the degradation of plant tissues (Grenet *et al,* 1989a,b). Furthermore, little work has been carried out on the interactions between fungi and rumen cellulolytic bacteria in fiber degradation and most studies have dealt with hydrogen transfer between fungal species and hydrogenotrophic bacterial species (Bauchop and Mountfort, 1981; Mountfort *et al,* 1982; Fonty *et al,* 1988; Joblin *et al,* 1989; Marvin-Sikkema *et al,* 1990). Because of their cellulolytic and hemicellulolytic activity (Orpin and Joblin, 1988; Fonty and Joblin, 1990), rumen fungi can either compete or, in contrast, act synergistically with cellulolytic bacteria to degrade the plant tissues ingested by ruminants.

The aim of this study was to compare dry matter (DM) loss of maize stem by *Piromyces* (formerly *Piromonas*) *communis*, a species with filamentous rhizoids, and by *Caecomyces* (formerly *Sphaeromonas*) *communis*, a species with bulbous rhizoids, in pure culture or in association with cellulolytic bacterial species, *Ruminococcus flavefaciens* or *Fibrobacter (Bacteroides) succinogenes* subsp *succinogenes*. Plant cell wall degradation by fungal monocultures was also studied by scanning electron microscopy.

MATERIALS AND METHODS

Origin and culture maintenance of the microbial strains

C communis FG10 and *P communis* FL came from the culture collection of our laboratory and were isolated from the intestine of a cow and a sheep rumen respectively. *F succinogenes* subsp *succinogenes* S85 (ATCC 19169) and *R flavefaciens* 007 were kindly provided by MP Bryant (University of Illinois, Urbana, IL, USA) and by CS Stewart (Rowett Research Institute, Aberdeen, UK), respectively.

Bacterial and fungal cultures were maintained in the medium described by Joblin (1981), to which 0.1% of solution I and 0.05% of solution II were added. Solution I: Ca D panthotenate 0.02%, pyridoxin 0.02%, riboflavin

0.02%, thiamin HCl 0.02%, nicotinamide 0.001%, folic acid 0.0005% and biotin 0.0005%, solution II: cobalamin: 0.00005%. The cultures were grown under CO_2 in Bellco tubes (Bellco Glass Inc, Vineland, NJ, USA) according to the method of Hungate (Hungate, 1969).

Evaluation of forage digestion

Dry weight loss of forage and end products of fermentation

The degradation and fermentation of ground maize stem (particle size 0.8 mm) by *C communis* FG10 and *P communis* FL were studied in monoculture or in coculture with *R flavefaciens* 007 or *F succinogenes* S85. Bacterial monocultures were also evaluated. Bacterial and fungal cultures were grown in tubes containing 10 ml of Joblin's medium without agar and in which cellobiose was replaced by maize stem (*Zea mays* L) as sole energy source (100 mg/tube).

The fungal inocula were composed of 0.5 ml of a 48-h-old culture on cellobiose (10^3–10^4 zoospores/ml) and the bacterial inocula of 0.5 ml of a 24-h old culture on cellobiose (10^8 bacteria/ml). Co-cultures were made by inoculating the 2 microorganisms simultaneously.

The extent of dry matter (DM) disappearance from maize stem was measured in triplicate after 2, 4, 6 and 8 d of incubation at 39 °C. Non-inoculated tubes were used as controls. At the end of culture, the tubes were centrifuged at 900 *g* for 15 min. The supernatant was removed for analysis of the end-products of fermentation and the pellet was washed 3 times with distilled water. The tubes were dried at 80 °C for 72 h. They were then weighed and the amount of substrate degraded was calculated.

The products of carbohydrate fermentation were analysed by gas chromatography (volatile fatty acids, ethanol and gas) (Jouany, 1982) and by enzyme assay (formate, L- and D-lactate) according to the method of Boehringer (Boehringer Mannheim France SA, Meylan, France).

Scanning electron microscopy (SEM) of tissue degradation

The maize stems (*Zea mays* L) were harvested at bloom. The internode bearing the ear was selected and cross-sections 3 mm thick cut halfway along it. The samples were stored at –15 °C. The degradation of the plant tissues was studied by placing 3 or 4 stem fragments in 10 ml of Joblin medium (1981), in which cellobiose was replaced by plant fragments as the sole energy source. Incubation times were 16, 24 and 48 h for *P communis* and 36, 72 and 96 h for *C communis*. Samples were also incubated for 24 h at 39 °C in non-incubated control tubes.

After incubation the plant fragments were rinsed in distilled water and then prepared for observation by SEM by the method previously described (Grenet and Barry, 1988) modified as follows: the substrates were fixed for 24 h at ambient temperature instead of 3 h, with 4% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.4.

RESULTS

Dry weight loss of forages

The maize stem was extensively solubilized in the medium, since there was a dry matter loss of 38% in the non-inoculated control cultures. The 2 cellulolytic bacteria degraded the maize stem at the same extent (about 60% DM loss in both cultures). However, the rate of degradation with *F succinogenes* was much faster than with *R flavefaciens*, as the former achieved maximum DM degradation after 2 d and the latter after 4 d.

After 8 d of incubation in monoculture, *P communis* was almost as effective as the bacterial monocultures (56% degradation) (figs 1 and 2). However, the fungus degraded the substrate much more slowly and maximum digestion was only reached after 6 d. In comparison with the other monocultures, *C communis* was the least able to degrade plant cell walls. Its rate of degradation was also much slower with 46% DM loss after 4 d of culture compared with 55, 58 and 61% for *P communis*, *R flavefaciens* and *F succinogenes* respectively (figs 3 and 4).

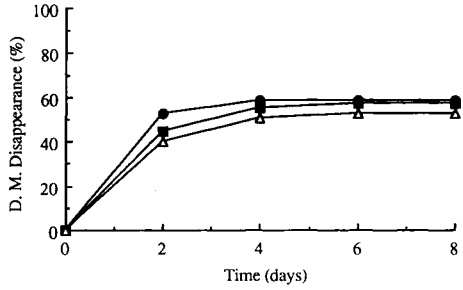


Fig 1. Maize stem degradation by *P communis* (■) and *R flavefaciens* (●) in axenic cultures or in co-culture (Δ). Values are the mean of triplicate cultures. SD are less than 5% of the means.

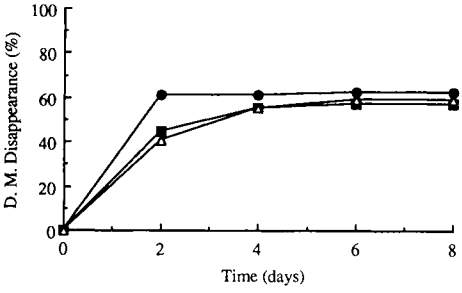


Fig 2. Maize stem degradation by *P communis* (■) and *F succinogenes* (●) in axenic cultures or in co-culture (Δ). Values are the mean of triplicate cultures. SD are less than 5% of the means.

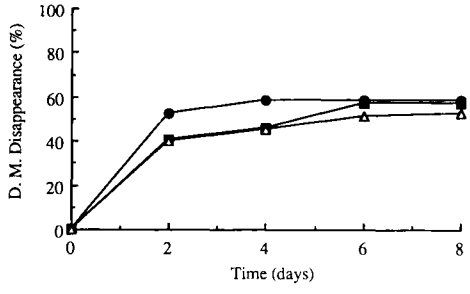


Fig 3. Maize stem degradation by *C communis* (■) and *R flavefaciens* (●) in axenic cultures or in co-culture (Δ). Values are the mean of triplicate cultures. SD are less than 5% of the means.

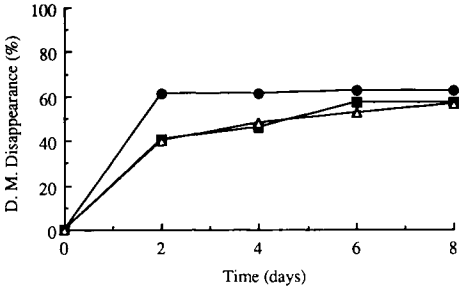


Fig 4. Maize stem degradation by *C communis* (■) and *F succinogenes* (●) in axenic cultures or in co-culture (Δ). Values are the mean of triplicate cultures. SD are less than 5% of the means.

The 4 co-cultures studied were less effective than the monocultures and followed the kinetics of degradation of the least effective microorganism, the fungus (figs 1, 2, 3 and 4). Unlike the bacterial monocultures and *P communis* in monoculture, the bacteria-fungi co-cultures degraded the substrate gradually; maximum degradation was only reached after 6 d of incubation with *P communis* in co-culture and after 8 d with *C communis* co-cultures.

The end-products of fermentation of the co-cultures were quantitatively close to those of the fungal monocultures. However, the production of acetate increased slightly and that of ethanol decreased slightly (tables I and II).

SEM observations

The phloem and parenchyma of samples incubated for 16 h with *P communis* were partially degraded (fig 5). A thick mass of rhizoids covered the samples. After 24 h of incubation the parenchyma was further degraded. After 48 h, what remained of the vascular bundles was observed clearly from the surface of the samples (fig 6). In

Table I. Maize stem fermentation products of *Piromonas communis*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in axenic cultures or in co-culture.

End-product [a]	P communis	R flavefaciens	F succinogenes	P communis + R flavefaciens	P communis + F succinogenes
Formate	249 ± 2	21 ± 1	11 ± 1	257 ± 2	244 ± 18
Acetate	188 ± 5	114 ± 4	83 ± 8	215 ± 8	203 ± 6
Propionate	0	13 ± 4	0	0	T [b]
Lactate (D + L)	288 ± 5	127 ± 1	0	173 ± 1	296 ± 5
Ethanol	11 ± 1	0	0	6 ± 2	6 ± 1
Hydrogen (%)[c]	17	8	0	18	20

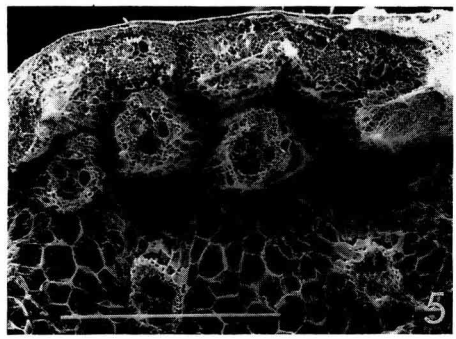
[a] Disappearance of DM expressed in µmol/100 µg (mean of 3 values ± SD); [b] only traces; [c] expressed in % of the total gas phase.

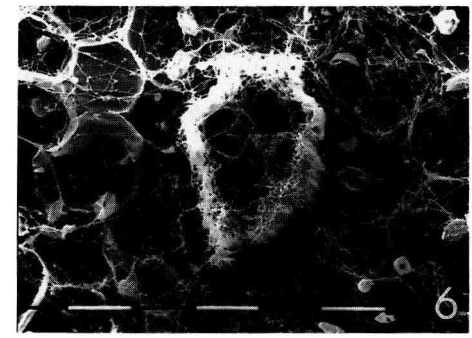
Table II. Maize stem fermentation products of *Caecomyces communis*, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes* in axenic cultures or in co-culture.

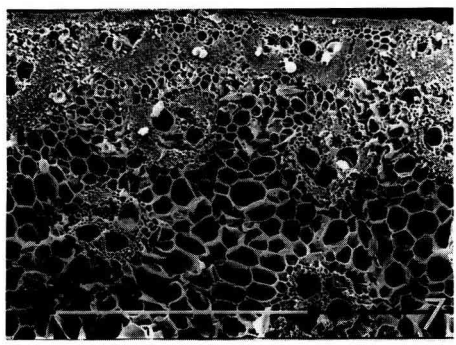
End-product [a]	C communis	R flavefaciens	F succinogenes	C communis + R flavefaciens	C communis + F succinogenes
Formate	21 ± 8	21 ± 1	11 ± 1	206 ± 3	184 ± 7
Acetate	151 ± 5	114 ± 4	83 ± 8	175 ± 8	181 ± 7
Propionate	0	13 ± 4	0	0	0
Lactate (D + L)	83 ± 1	127 ± 1	0	74 ± 3	60 ± 1
Ethanol	10 ± 2	0	0	T [b]	6 ± 1
Hydrogen (%)[c]	21	8	0	21	20

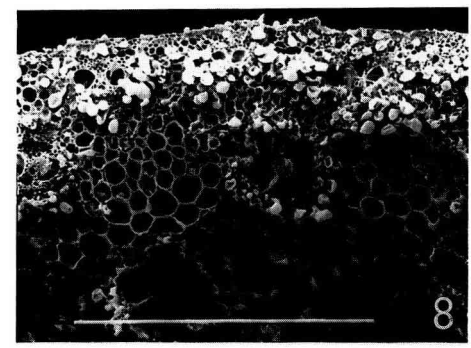
[a] Disappearance of DM expressed in µmol/100 µg (mean of 3 values ± SD); [b] only traces; [c] expressed in % of the total gas phase.

contrast, degradation by *C communis* was far less noticeable. This was due to the fact that the fungus produces no filamentous rhizoids. After 36 h of incubation, the rhizoids were reduced to vesicles attached mainly to lignified tissues of the maize stem (fig 7). At this stage, the tissues of the phloem and parenchyma were little degraded. The extent of degradation was not much greater after 72 h. There was a fairly large number of vesicles attached to samples incubated for 96 h in *Caecomyces* cultures. The phloem (fig 8) and parenchyma were degraded, causing the vascular bundles to project slightly from the surface.









Figs 5–8. 5. Maize stem incubated for 126 h with *Piromyces communis* observed by SEM. The phloem and parenchyma are degraded. Bar = 1 mm. **6.** Maize stem incubated for 48 h with *Piromyces communis* observed by SEM. The vascular bundles are projecting from the surface of the parenchyma. Bar = 0.1 mm. **7.** Maize stem incubated for 36 h with *Caecomyces communis* observed by SEM. The fungal vesicles are attached to the sclerenchyma and the fibers of the plant fragment. Bar = 1 mm. **8.** Maize stem incubated for 96 h with *Caecomyces communis* observed by SEM. Numerous vesicles are attached to the sclerenchyma and fibers. The phloem and parenchyma are partially degraded. Bar = 1 mm.

DISCUSSION

Unlike the observations of Bernalier *et al* (1988) on the degradation of cellulose filter paper by *P communis*, our findings showed that the cellulolytic bacteria *R flavefaciens* and *F succinogenes* had a greater ability to degrade maize stem than the 2 fungal species studied. The discrepancy between results may be due to the nature of the media used.

P communis degraded maize stem faster than *C communis* (55 as against 45% of substrate degraded after 4 d of culture). Electron microscopic observations confirmed this greater effectiveness of degradation as *C communis* was seen to colonize the substrate much later than *P communis*. Our results are in agreement with those of Gordon and Ashes (1984), Orpin (1983, 1984) and Bernalier *et al* (1991), who have already shown that the

fungal species possessing rhizomycelia (*Neocallimastix* and *Piromonas*) degrade plant cell walls more effectively than those with vesicles *(Caecomyces).* The filamentous rhizoids of *P communis* allow it to penetrate deep into the plant tissues and thereby increase its hydrolytic activity. Bauchop (1979) and Ho *et al* (1988) have shown that filamentous rhizoids can penetrate plant tissues up to a depth of 450 μm. As a result of their hydrolytic activity against the polysaccharides of the plant cell walls they modify the mechanical resistance of the tissues (Akin *et al,* 1983; Akin *et al,* 1989). In addition, Joblin (1989) observed that the action of *C communis*, which produces only bulbous rhizoids, is both physical and enzymatic. As the bulbs expand, pressure is exerted on the surrounding tissues so that the plant fibers are shredded, creating a large surface for the microbial enzymes to attack. Like the other rumen fungal species, *C communis* colonizes preferentially lignified tissues (sclerenchyma, vascular bundle fibers). This preferential colonization of tissues that are highly resistant to degradation is not the result of competition with bacteria for more digestible tissues since the phenomenon has also been observed in axenic cultures. It can only be assumed that these microorganisms have a particular ability for colonizing and degrading tissues with lignified cell walls as suggested by Windham and Akin (1984) and Akin and Benner (1988). However, it is not possible to affirm from this study that they are able to solubilize lignin.

The difference in the effectiveness of degradation of cellulosic substrates generally observed between *C communis* and *P communis* was less marked with maize stem. This may be because the substrate was highly solubilized in the culture medium. In control cultures, large amounts of reducing sugars were found in the medium (data not shown). They probably enhance the growth of *C communis* and thereby improve dry matter losses of the substrate by increasing the number of fungal cellulases. The smaller difference in effectiveness may also have been due to the fact the better degradation of plant cell walls by *P communis* compared to *C communis* was limited by the high solubilization of maize stem.

In contrast to the results observed with methanogenic bacteria (Bauchop and Mountfort, 1981; Mountfort *et al,* 1982; Fonty *et al,* 1988; Joblin *et al,* 1989; Marvin-Sikkema *et al,* 1990), the association of *P communis* FL and *C communis* FG10 with the cellulolytic bacteria did not increase the amount of substrate degraded. Likewise, no metabolic interaction was observed, and the fermentative pattern observed in the co-cultures was very similar to that of the fungal monocultures. It is interesting to observe that the effectiveness in cellulose degradation of all co-cultures is slightly lower than that of the best degrader, which suggests a slight competition between the 2 organisms. The increase in acetate production observed in co-culture is difficult to interpret since the 2 microbes are acetate-producing microorganisms. However, this increase in acetate is worthwhile for the ruminant if it appears *in vivo.*

The antagonistic effect of *R flavefaciens* against rhizoidal fungi (*P communis* and *N frontalis*) observed by Bernalier *et al* (1988) on cellulose filter paper was not recovered in this study. Richardson *et al* (1986) an Irvine and Stewart (1991) noted an inhibitory effect of *Ruminococci* on fungal cellulolysis. According to Bernalier and Fonty (unpublished data), this may be due to the secretion by the bacteria of a protein factor that inhibits fungal cellu-lases. Therefore, the interactions between bacteria and cellulolytic fungi in the rumen seem to vary depending on the substrate.

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