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Detection and discrimination between ochratoxin producer and non-producer strains of *Penicillium nordicum* on a ham-based medium using an electronic nose

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Abstract

The objective of this study was to evaluate the potential use of volatile fingerprints produced by *Penicillium nordicum* to determine whether strains were producers or non-producers of ochratoxin A on a ham-based medium. Studies were thus carried out on a 3% ham medium inoculated with spores at two water activities (0.995, 0.95 a_w) at 25°C for up to 14 days. A spore lawn inoculation technique was employed and agar discs were destructively sampled after 1, 2, 3, 7 and 14 days, placed in 30 ml vials, sealed and the head space analysed after 1 hour incubation. The qualitative response of a hybrid sensor array of 23 sensors was used to try and discriminate between controls, OTA producing and non-producing strains of *P. nordicum*. The effect of the environmental conditions on growth and OTA production were also evaluated. Principal Component Analysis was used to analyse the volatile fingerprint data sets. Up to 72 hrs high associations were observed based on a_w more than the capacity for OTA production. However, after 7 days incubation the hybrid sensor array was able to discriminate the two strains grown at 0.995 a_w. After 14 days growth the hybrid sensor array was able to discriminate the OTA producers from the non-producer strain replicates and the control ham medium at both a_w levels.

Keywords: moulds; mycotoxin; electronic nose; dry-cured ham; volatile patterns

Introduction

Stored ham is commonly covered by mould whose type and extension depends on ecological conditions in the storehouse, especially the prevailing temperature and humidity (Spotti et al., 1999). The microbial community is important as it determines the aroma and the organolectic aspects of the product, and contributes to the characteristics of final products

in different geographic regions (Thanks et al., 1986; Leistner, 1986); unfortunately, some component microorganisms can also cause deterioration or produce undesirable metabolites.

Results from studies in various countries have shown that the most important source of natural toxin contamination for human consumption of such products is from ochratoxin A (OTA), when compared with other products of animal origin (Govaris et al., 2007). The kidney and liver are the main target organs of OTA, resulting in teratogenicity, carcinogenicity, and mutagenicity (Lai WeiHua et al., 2009; Govaris et al., 2007); it is also implicated in the aetiology of Balcan Endemic Nephropathy (EFSA, 2006).

The presence of the toxin in meat and meat products could be the result of either direct contamination with moulds or carry over from animals exposed to naturally contaminated feed (Gareis, 1996). Recent data supports the direct contamination as a major source (Battilani & Pietri, unpublished data). *Penicillium nordicum*, responsible for the production of OTA in proteinaceous food, has been observed sporadically (Spotti et al., 2001) or as an important component (26%) of the total *Penicillium* population isolated from ham in Italy (Battilani et al., 2006).

The presence of *Penicillium* species as the dominant genus (88.3%) on dry-cured pork meat was also reported in Norway (Asefa et al., 2009), with *P. nalgiovense*, *P. solitum* and *P. commune* as the main species isolated, while in Spain the mould flora was dominated by *Aspergillus* spp. and primarily by *A. laucus*, *A. fumigatus*, *A. niger* and *A. flavus* (Rojas et al., 1991). No reports of *P. nordicum* on stored pork meat are available, but possibly some misidentification of *P. verrucosum* could have occurred (Iacumin et al., 2008).

Mycotoxins and other non-volatile metabolites have traditionally been detected using high performance liquid chromatography coupled to diode array detection (HAPLC-DAD) and often also to mass spectrometry (LC-DAD-MS) (Smedsgaard, 1997). A new validated HPLC-FLD method for OTA detection in dry cured meat and in blue cheese has been developed recently to minimise the matrix effect and to improve the analysis performance (Dall'Asta et al., 2007). These methods are destructive: they are applied at the end of the ham ripening period and can thus lead to a loss of commercial product.

There has been interest in using volatile production patterns to try and discriminate between spoilage fungi contaminating food products (Magan and Sahgal, 2007). Recently, Sahgal et al. (2007) demonstrated the potential of discriminating between toxigenic and non-toxigenic strains of mycotoxigenic species by using volatile production patterns. Previously, Keshri amd Magan (2000) were able to discriminate fumonisin and non-fumonisin producer

strains of *Fusarium verticillioides* based on volatile fingerprints. They also found that this gave earlier results than those based on quantitative enzyme assays.

The objective of this study was to evaluate the potential for discrimination between a toxigenic and non-toxigenic strain of *P. nordicum* using volatile production patterns on a ham-based medium and the effect that environmental conditions had on growth and toxin production.

Materials and methods

Strains used

Two strains of *P. nordicum* were included in the study, a OTA producer (MPVP P1669, BFE 838) and a non-OTA producer (MPVP P1446, BFE 851) (Bogs et al., 2006). They are both held in the fungal collections of the Institute of Entomology and Plant Pathology, UCSC in Piacenza (Italy) and Federel Research Centre for Nutrition and Food, in Karlsruhe (Germany).

Inoculum preparation

The strains were initially inoculated on Yeast Extract Agar (Peptone from casein (tryptone) 6.0 g; yeast extract 3.0 g; agar-agar 15.0 g) and incubated at 25°C for seven days for the inoculum preparation. The spores were collected using 10 ml of sterilised water and gently agitating the culture surface to remove conidia. The concentration of the suspension was modified to 10^8 conidia/ml by initially determining the concentration using a haemocytometer.

Growth studies on ham medium (please elaborate ham medium preparation)

The medium used in these studies was based on 3% freeze dried ham per 1000 ml water with the addition of 2% technical agar. This basic medium was modified to different a_w levels by substituting water with glycerol/water solutions (Dallyn and Fox, 1980) to obtain 0.98-0.80. This a_w range was used for the growth and OTA experiments. The treatments were incubated at 25°C and the diameter of the colonies in two directions was measured after 2, 5, 7, 9, 12 and 14 days. Three small portions of medium (0.5 mm diameter) were collected from each plate and used for OTA analysis at the end of the incubation period (14 days).

Volatile production patterns of P. nordicum strains

For the detection of volatile fingerprints, experiments were carried out on unmodified medium (0.995 a_w) and at 0.95. The media were sterilised and the molten mixtures poured into 9 cm Petri plates. For volatile production patterns a 0.2 ml conidial suspension was spread over the whole surface of each of up to 24 replicate plates. These were incubated and four replicates removed and destructively sampled.

Three random agar plugs (1 cm diameter) were taken from each replicate and placed in 30 ml head space analyses vials after 1, 2, 3, 7 and 14 days and sealed with screw caps and septa. The samples were incubated at 25°C for 1 hour to allow a build up of volatiles and then analysed.

Volatile analyses was done in an NST 3220 Lab Emission Analyser carousel (Applied Sensors, Linköping, Sweden). This system contains 10 metal oxide semiconductor field effect transistors sensors (MOSFET), 12 metal oxide semiconductors sensors (MOS), and a humidity sensor. It employs an auto sampler and a robotic double needle system to draw sample headspace from the sampling vial, and flush it over the hybrid sensor array and the headspace is passively replaced with air through the 2nd injection needle.

Data analysis

Data on fungal growth, OTA production and the sensor responses were analysed with the Univariate analysis of variance in SPSS (Statistical Package for Social Science, ver. 15.0, 2006 SPSS Inc., Chicago Il USA) and a Tukey test was applied to separate means.

Data collected with the electronic nose were analysed by in-built software package furnished by the system; Principal Component Analysis (PCA) and Cluster Analysis (CA) were applied. Statistica 7 was used for CA where the most representative e-nose sensors, using those showing significant differences in their response were included. Euclidean distance was selected to establish similarities between samples and Ward's method to aggregate distances.

Results

Effect of environmental factors on growth and OTA production

Growth of *P. nordicum* was slow with initial visible growth after 2 days incubation and, by the 14th day, the diameter of colonies was only about 27 mm (Table 1). The colonies were significantly larger at 0.98 and 0.95 a_w compared to lower a_w treatments.

Table 2 shows the OTA production after 14 days incubation. The highest amount of OTA was detected at 0.98 a_w, followed by 0.95. The amounts obtained were significantly higher when compared to the other conditions.

Discrimination between P. nordicum strains based on volatile fingerprints

Figure 1 shows the relative response of one set of sensors to the different treatments used in this study after 7 days incubation. There were differences in the response of the sensor array to the toxigenic and non-toxigenic strain of *P. nordicum* which implies a difference in volatile production patterns.

The PCA analyses after 1 – 3 days suggested very little discrimination between treatments perhaps because of the very slow growth of the *P. nordicum* strains. However, after 7 days there was discrimination of the *P. nordicum* strains, especially on the unmodified ham medium (0.995 a_w, Figure 2). More than 90% of the variation was accounted for by PCA 1 and 2. This was confirmed by the cluster analyses which showed the spatial separation between treatments (Figure 3).

Figure 4 shows the results of the PCA after 14 days. This shows the effective discrimination between the *P. nordicum* producer and non-producer strains at both a_w levels examined. However, producers grown at 0.995 and 0.95 a_w could not be effectively differentiated and this was confirmed by CA (data not shown).

Discussion

The application of an e-nose in detecting the presence of *P. nordicum* and OTA based on volatile fingerprints gave promising results. The e-nose was able to discriminate the OTA producer strain with respect to the non-producer after 7-14 days incubation. The volatile fingerprints were also slightly different depending on the water availability of the medium after 14 days. This may partially be due to the biosynthetic pathways for OTA production being more active in the OTA producing strain (MPVP1669) at 0.98 than 0.95 a_w.

The use of volatile fingerprints to separate between the two strains after 7 days incubation was limited, and was possible under conditions with freely available water (0.995 aw). At 0.95 a_w the slow growth and volatile production patterns may have been lower resulting in the non-discrimination between the strains. Previous work with *P. nordicum* grown on artificial media and incubated at 20°C for 7 days produced approx. 6 ppb of OTA (P. Battilani, unpublished data). It may be that the parallel production of volatiles may be to low for effective discrimination using our approach.

Sahgal et al. (2006) obtained similar levels of discrimination between strains of dermatophytes when the concentration of volatiles generated in the headspace or the types of volatiles were different. Needham and Magan (2003) in work relevant to the present study, found some discrimination between toxigenic and non-toxigenic strains of *P. verrucosum* in

relation to ochratoxin production at 0.95 a_w on wheat-based agar media, although results for A.flavus were less clear. Recent work on strains of the *Aspergillus* section *nigri* species has shown more promise (Cabanes et al., 2009).

Previous studies with food spoilage microorganisms has tried to take into account the volatile profiles produced and analysed by an e-nose combined with mass spectrometry (GC-MS) . This showed a good correlation between volatiles identified during the development of the spoilage profile (Olsson 2000; Tognon et al., 2005; Bianchi et al., 2009). An interesting application of e-nose was reported by Karlshoj et al., (2007). They developed a classification models for *P. expansum* spoilage of apples and a prediction models for patulin concentration usable for apple juice production. This was based on the correlation between e-nose data and HPLC quantification of patulin. The studies by Keshri and Magan (2000) certainly showed that there was potential for discriminating between fumonisin and non-fumonisin producing strains of *F. verticllioides* in vitro. Indeed, the volatile fingerprint approach was shown to be faster than other more traditional approaches. The potential for this approach ahs also been more recently reviewed by Sahgal et al. (2007).

This paper has shown that potential exists for using this approach. More detailed work is now required with *P. nordicum* and OTA production in ham and pork cured meat products to evaluate whether the relationship between volatile production patterns and threshold levels of OTA can be correlated to enable rapid and real time predictions to be made of the presence of harmful strains of *P.nordicum* or harmless strains which do not represent a risk to consumers.

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Table 1. Effect of incubation time and available water on the growth of *P. nordicum*, measured as diameter of the colonies on a 3% ham medium at 25oC. Figures with different letters are significantly different (P=0.05).

	Diameter (mm)	
Incubation time (days)		
2	4.6	f
5	11.9	e
7	15.6	d
9	19.2	c
12	24.3	b
14	27.3	a
Available water		
0.98	30.1	a
0.95	32.1	a
0.93	20.4	b
0.90	12.5	c
0.85	3.0	d
0.80	3.0	d

Table 2. The relative amounts of ochratoxin A produced by colonies after 14 days incubation with the concomitant colony diamters. Figures with different letters are significantly different (P=0.05)

a_w	**Diameter (mm)**		**Ochratoxin A (ng/g)**	
0.98	47.6	a	7992.7	a
0.95	51.3	a	4458.2	b
0.93	33.0	b	1390.1	c
0.90	23.0	b	481.7	c
0.85	3.0	c	0.0	c
0.80	3.0	c	0.0	c

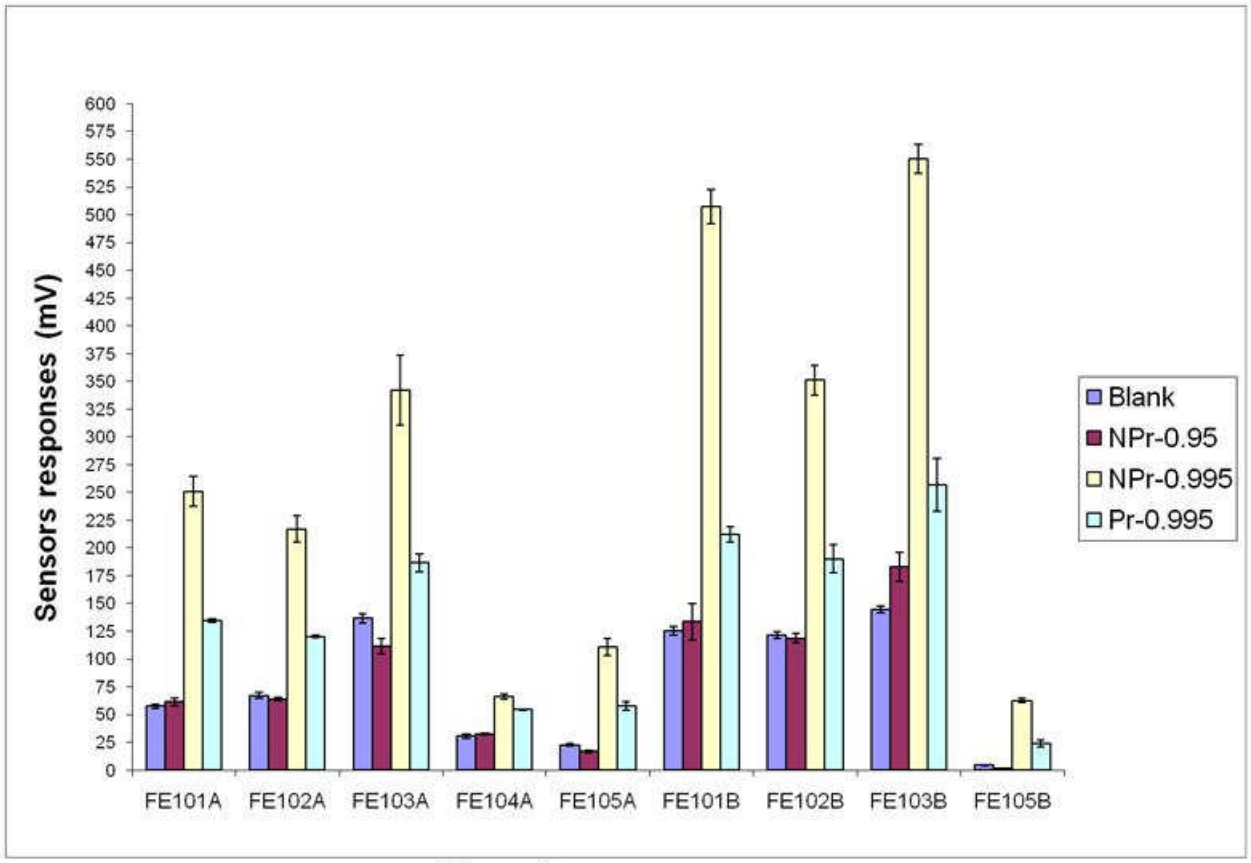

Figure 1.

Figure 1. Example of the mean response of the metal oxide sensors to volatile produced by different treatments for both the toxigenic (MPVP1669, BFE 838) and non-toxigenic (MPVP1446, BFE 851) strains after 7 days at 25^{o}C on a 3% ham-based medium.

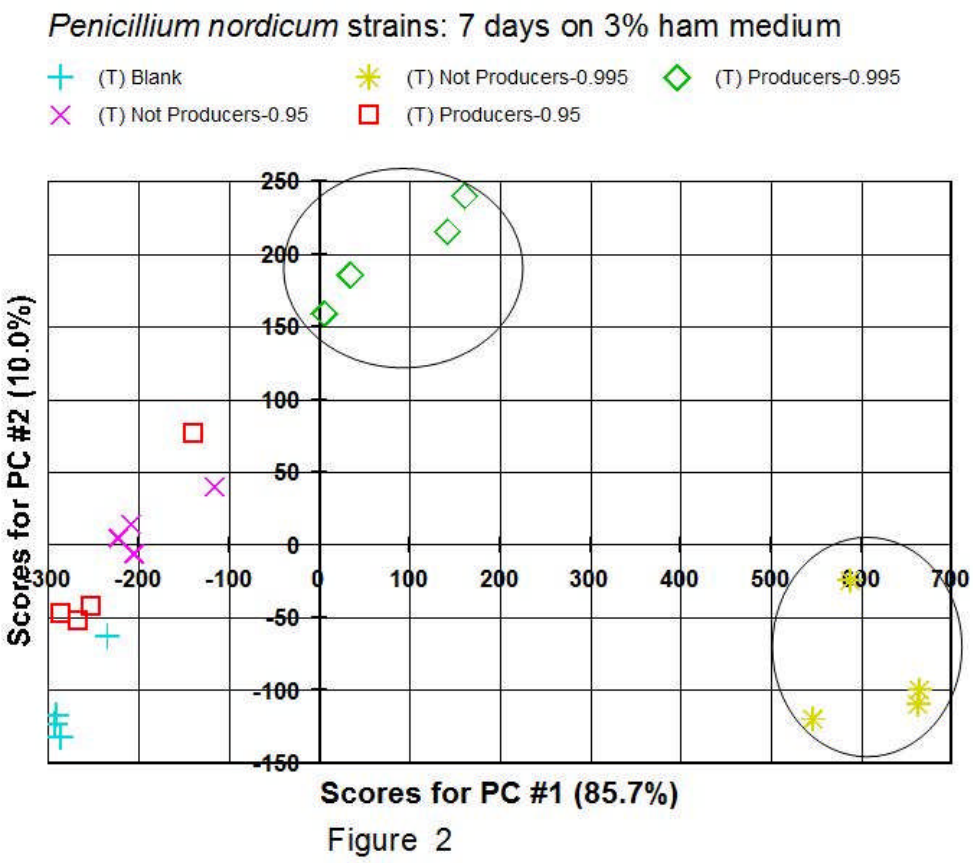

Figure 2

Figure 2. The PCA plot of the data after 7 days incubation to show that there is some discrimination between the *P. nordicum* strains on a 3% ham-based medium based on the volatile production patterns.

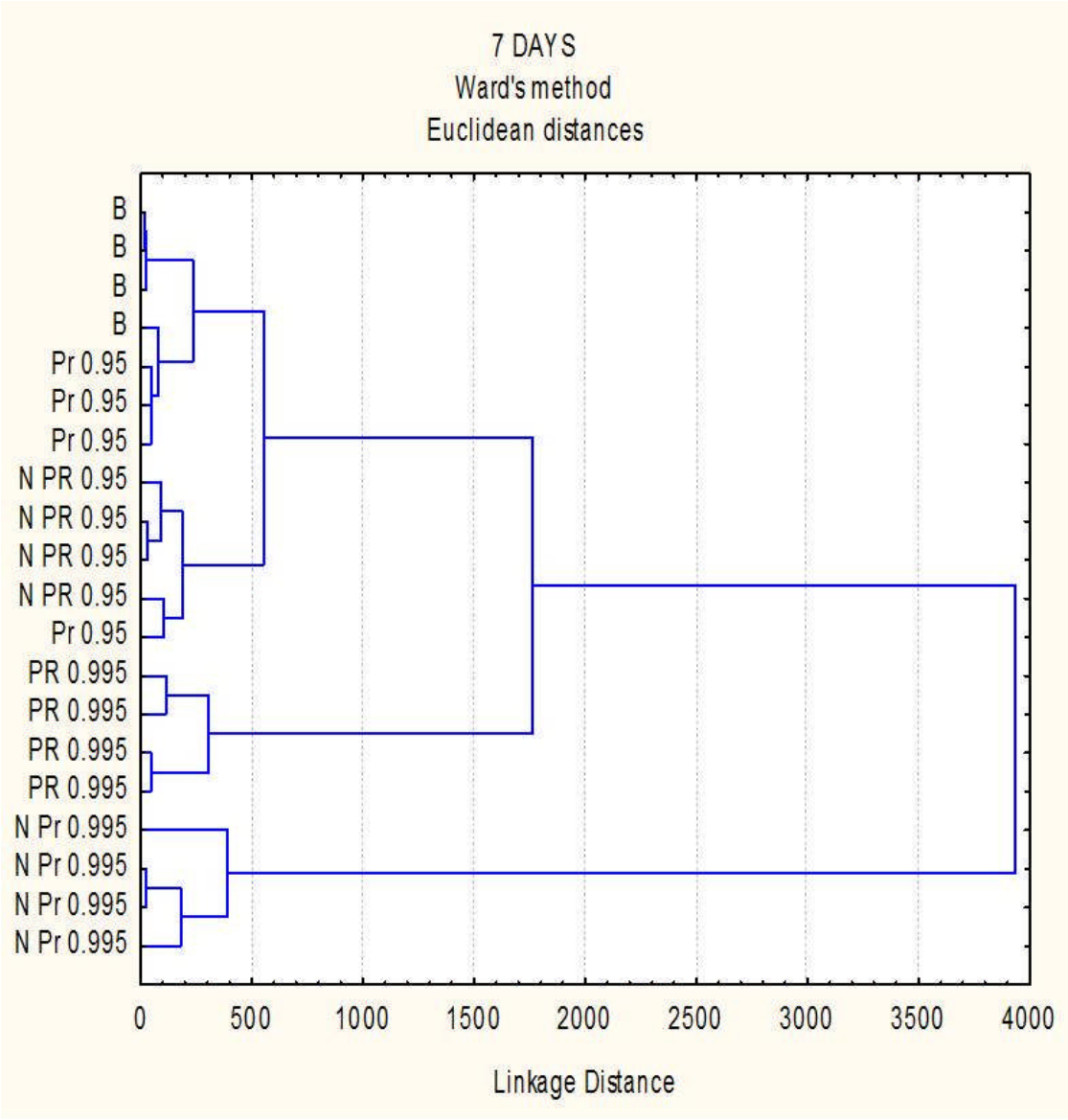

Figure 3. Cluster analyses after 7 days. Key to treatments: **B**=blank; **N PR 0.99**=non-producer at 0.99 a_w; **N PR 0.95**=non-producer at 0.95 a_w; **PR 0.99**=producer at 0.99 a_w; **PR 0.95**=producer at 0.95 a_w.

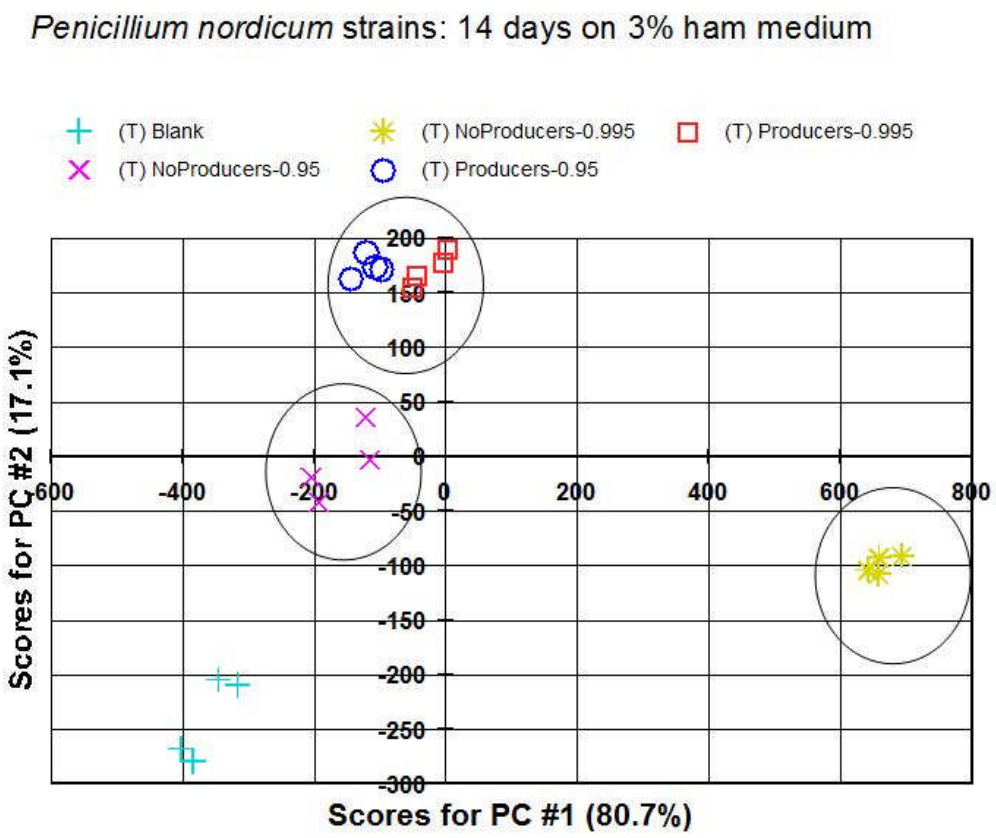

Figure 4

Figure 4. PCA after 14 days showing the relative discrimination between treatments. Key to treatments: **B**=blank; **N PR 0.99**=non-producer at 0.99 a_w; **N PR 0.95**=non-producer at 0.95 a_w ; **PR 0.99**=producer at 0.99 a_w; **PR 0.95**=producer at 0.95 a_w.