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Influence of N,N-bis(3-aminopropyl)dodecylamine on the Mycelium Growth and the Cell Wall Composition of Resistance and Sensitive Strains Belonging to the Genus *Aspergillus*

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A b s t r a c t

Resistance causes of moulds to N,N-bis(3-aminopropyl)dodecylamine (APDA) for selected species of *Aspergillus niger* and *Aspergillus flavus* was examined. Control (sensitive) strains and resistant strains, cultured at 0.05% triamine, were used in the experiments. The non-resistant strains did not have growth capacity in this amount of ADPA while the resistant strains were characterised by a smaller biomass increase. Individual stages of the development of the mycelium occurred later than those in the control samples. The participation of the cell wall in the mycelium biomass of the resistant strains was higher by 7.5%. The glucan content in the wall dry mass was lower by 11% than that in the sensitive strains. A 41% increase in the lipid content was recorded in the cell wall of resistant *Aspergillus flavus*. A 21% protein increase occurred in the wall of *Aspergillus niger* comparing to the control strain. Infrared spectrophotometric analysis of the cell wall did not reveal the presence of triamine. Most absorption bands disappeared in the wall of *Aspergillus flavus* while no additional absorption bands were registered in *Aspergillus niger*; some bands were only stronger than those in the control sample. The resistant strains were characterised by a smaller ergosterol content, the main constituent of cell membranes. Spectrophotometric analysis of the mycelium did not reveal significant qualitative changes; only quantitative changes were observed. It was noticed that the resistance reaction did not occur with the same intensity in both species studied. The resistant strain of *Aspergillus niger* was characterised by a slightly more intensive absorption within its entire spectrum range in comparison to control strain. In case of *Aspergillus flavus* the absorption was higher for control strain.

K e y w o r d s: moulds, resistance to disinfectants, N,N-bis(3-aminopropyl)dodecylamine, cell wall, ergosterol

Introduction

Various anti-microbial agents are used in the pharmaceutical, cosmetic, paper and food industries as well as in medicine. Many products used so far have become less effective as a result of spontaneous cellular mutation or phenotypic adaptation to harmful environment conditions. Most studies on the mechanisms of microbial resistance or inactivation have until recently concentrated on bacteria; fungi, however, characterised by high adaptability to physical and climatic environment conditions that allows them to colonise new substrates and to invade the human environment, should also be examined to identify their resistance.

A number of basic targets for antifungal agents are distinguished in a single cell (Groll *et al.*, 1998; Wills *et al.*, 2000). The cell wall, responsible for the cell shape, and the first contact point with the biocidal agent, is one of them. Chitin, glucan, mannan (these polysaccharides constitute 80–90% of the cell wall dry mass) as well as proteins (ca 20%) and lipids (1–3%) occur in the cell wall in most species (Farkaš, 1990; Fiema, 1994). It may be destroyed by inhibiting the synthesis of these structural components in the cell or by degrading the existing constituents of the wall.

The cell membrane, consisting of phospholipids, sphingolipids, sterols and proteins, is another target for disinfectant agents. Ergosterol, also called provitamin D_2, is the main sterol in the cell of moulds. It plays

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a very important role in the membrane of the cytoplasmatic wall in fungi as it is responsible for its appropriate functioning, including substance transport. The mechanisms of the action of antifungal disinfectants and biocidal agents as well as the mechanisms of fungal resistance have been examined only in few studies and still little is known about them (Strzelczyk, 2001).

The aim of the present study was to explain the presumable mechanism of the resistance of selected mould species belonging to the genus *Aspergillus* to N,N-bis(3-aminopropyl)dodecylamine (APDA). Researches were concentrated on mycelium growth dynamics assessment, cell wall composition, and ergosterol content of resistant and sensitive (control) strains. Moreover, based on infrared spectrophotometric analysis, the article gives the answer if triamine presence results in new bonds creation in biological material. The compound is a component of cleaning agents and disinfectants used in hospitals, the food industry and the cosmetic industry. It is used, for instance, in Australia, Germany or Ireland in agents such as Lonzabac, Gigasept or in bactericidal hand soap, KM 801 (Dibo and Brasch, 2001; web pages[1]).

N,N-bis(3-aminopropyl)dodecylamine (APDA) belongs to the group of triamines, and is a derivative of fatty amines with two free amino groups and a 12-carbon aliphatic chain.

Experimental

Materials and Methods

Fungal strains and culture conditions. Strains of *Aspergillus niger* Ł 0439 and *Aspergillus flavus* Ł 0422, deposited in the Collection of Pure Cultures at the Institute of Fermentation Technology and Microbiology, Technical University of Łódź, LOCK 105, as well as their varieties resistant to triamine, obtained in successive passages (the authors' unpublished studies), were the biological material.

Culture medium: nutrient solution M_0, pH = 6.2; containing 3% (w/v) of glucose, 0.3% (w/v) $(NH_4)_2SO_4$; 0.1% (w/v) KH_2PO_4; 0.5% (w/v) $MgSO_4 \times 7H_2O$; 0.5% (w/v) yeast extract. Culture conditions: temperature – 28°C, time – 7 days. Resistant strains were cultured on the M_0 solution to which 0.05% (w/w) triamine was added (89 ml culture medium + 10 ml APDA). Non-resistant control strains were cultured on M_0 without APDA (99 ml culture medium). Samples prepared as described above were treated with 1ml conidial suspension of sensitive or control strains (10^7 spores/ml).

Mycelium analysis comprised estimation of participation of the cell wall dry mass in the mycelium, chemical composition of the cell wall, spectrophotometric analysis of the mycelium and the walls.

The results given below are the arithmetic mean of three series of experiments.

Growth curves. After 1, 2, 3, 4, 5, 7, 8, 10, 12 days of culture, the samples were taken out from incubator, and washed with distilled water on the filter to separate mycelium from nutrient solution. Next, the mycelium was dried to constant mass, first at 30°C, next at 105°C.

Cell wall isolation. The mycelium was hydrolysed with 1% (w/v) dodecyl sulphate solution in volume of 100 ml for each 10 g of wet mycelium biomass (Kisser *et al.*, 1980). Then the mycelium was separated from the post-reaction mixture and washed with distilled water to remove cell protein structures. In order to check total flush, the absorbance of final filtrate was measured at wavelength $\lambda = 260$ nm. Cell walls were dehydrated with cooled absolute alcohol and dried to constant mass. The results were given as percentage of cell wall mass to total dry mass of mycelium.

Glucan and chitin determination. The cell wall was hydrolysed using 3 M HCl to obtain cell wall monomers (Rokem *et al.*, 1986). The total content of reducing sugars was determined in 1ml of the obtained hydrolysate using the colorimetric method with 3,5-Dinitrosalicyl acid (DNS) using spectrophotometer at wavelength $\lambda = 540$ nm. Acid was removed from the remaining hydrolysate using a Dowex1 anion exchanger. Determination consist in colour reaction between reducing sugars, DNS, and sodium-potassium tartrate. This method is characterised by high sensitivity – 0.1 mg of sugar at 1 ml. A Dowex50Wx4 cation exchanger was used to elute glucosamine from the sample studied. The eluate was concentrated to a defined volume under reduced pressure. The concentration of reducing sugars (glucan), expressed as the glucose content, was determined in this sample using the colorimetric method with DNS. The amount of glucosamine (chitin) was determined from the difference between the total number of reducing sugars and the amount of glucose obtained in the eluate. The results are given in mg per 1g of the cell wall dry mass.

Determination of the total amount of proteins. The Kjeldahl method was used to determine the protein content. As described in the method, nitric organic substances are converted into ammonium salts, and ammonia is distilled to the standard acid solution. The results are given in mg per 1g of the cell wall dry mass.

Lipid determination. The cell wall was suspended in distilled water and a mixture of chloroform and methane (1:2) was added (Kiejts, 1975). The extraction was conducted twice, a chloroform layer was separated, diluted with a mixture of chloroform and water (1:1), and shaken in a separatory funnel. The chloroform layer was transferred to a weighing bottle and evaporated to dryness. The results are given in mg per 1g of the cell wall dry mass.

Spectrophotometric analysis in the infrared. Mycelia and cell walls of both strains were dried to constant mass and extracted using CCl_4 after a 7-day culture. The extract was analysed using infrared spectroscopy (FTIR). Spectra were registered (FTIR Jasco 610) in the range 5000–400 cm^{-1} from the resolution 2 cm^{-1} in KSR-5 trays, 1.02 mm thick. The measured absorption value in the ranges characteristic of the compounds studied was linearly proportional to their concentrations.

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[1] Australian Government Department of Health And Ageing NICNAS web page: http://www.nicnas.gov.au/PUBLICATIONS/CAR/NEW/NA/NASUMMR/NA0100SR/na180.asp; Kitchenmaster NI Ltd web page: http://www.kitchenmaster-ni.com/safetyinfosheets/KM801.DOC

Ergosterol determination. The content of this component in the mycelium dry mass was determined after day 5 and day 7 of the culture using the spectrophotometric method (Gutarowska and Żakowska, 2002). The preliminary lipid extraction from the mycelium was conducted using methanol, and the samples were centrifuged. The unsaponifiable fraction was separated. The entire sample was boiled under a reflux condenser, and the cooled sample was extracted with hexane twice. The hexane fraction was evaporated to dryness. The dry residue obtained was dissolved in methanol and examined using spectrophotometry at wavelength $\lambda = 282.6$nm. The results are given in mg of ergosterol per 1g of the mycelium dry mass.

Statistic. Anova test were used in order to verify if obtained data are statistically essential. Test depends on variation analysis. Verification was performed in Origin, data analysis and technical graphics software.

Results

Development of the mycelium. The results of the comparative analysis of the development of resistant strains, cultured in the presence of 0.05% of N,N-bis(3-aminopropyl)dodecylamine, and that of non-resistant strains growing in control conditions, are discussed in this part. The non-resistant strains did not show growth capacity at this amount of ADPA.

The control strain was characterised by a greater biomass increase in comparison with the resistant strain, cultured in the presence of triamine, already in the the first day. The greatest differences are noticeable throughout first and second day, and also between fifth and seventh day of the experiment. The addition of triamine to the resistant strain culture suppressed its growth, so the growth phases were prolonged (Fig. 1).

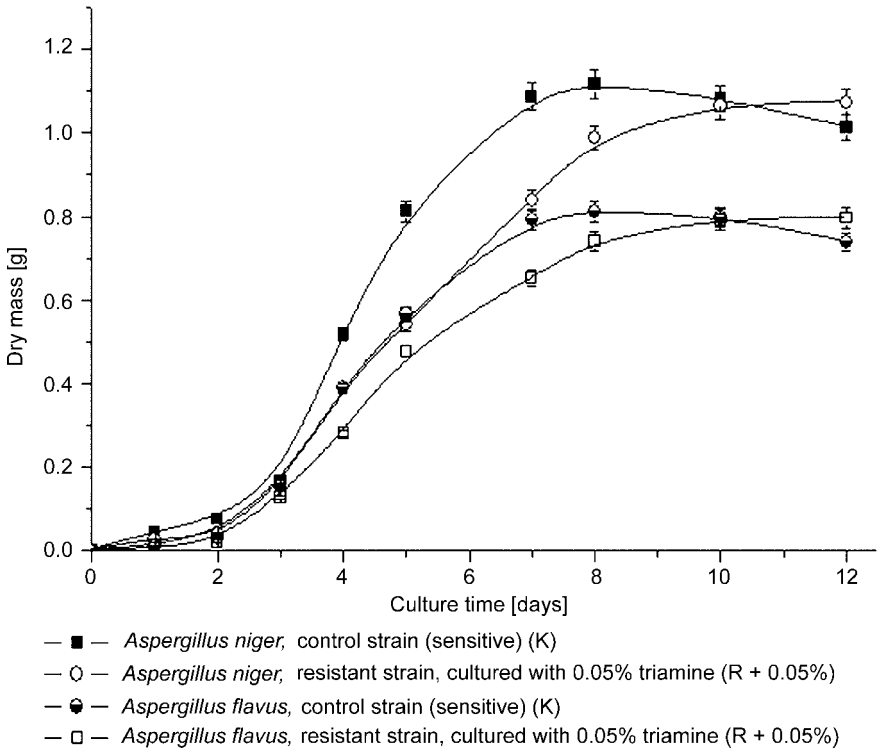

Fig. 1. Growth of *Aspergillus* strains sensitive and resistant to triamine

The control strain entered the stationary phase on the seventh day, and the amount of its biomass began to decrease from tenth day. The stationary phase for the resistant strain did not begin until eighth day. The growth curves for the *Aspergillus flavus* strain were similar to those of *Aspergillus niger*; the biomass yield, however, was smaller.

The greatest biomass differences between the resistant strain, cultured in the presence of 0.05% triamine, and the control (sensitive) strain occurred between day 1 and day 2, as well as on day 4 and day 7. The slope of the growth curve of the resistant strain is slightly greater. The vegetative growth phase of this strain was prolonged, and the stationary phase began around day 8, what is two days later than in control culture.

Cell wall in the sensitive strains and the resistant strains. The cell wall content in the mycelium dry mass of the sensitive strains and the resistant strains is shown in Figure 2. For the resistant strains of both species belonging to the genus *Aspergillus*, the cell wall content in the mycelium dry mass in the presence of

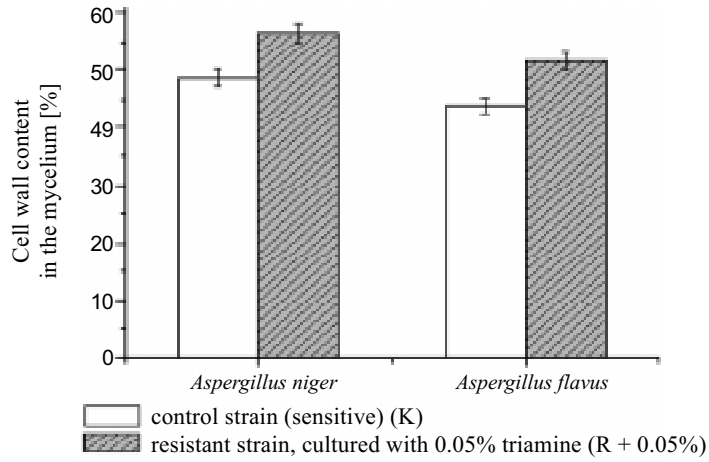

Fig. 2. Participation of the cell wall in the mycelium after 7 days of culture

0.05% triamine exceeded 50%. The non-resistant strains, growing in control conditions, were characterised by the cell wall content in mycelium dry mass lower by *ca* 7.5% than that in the resistant strains, cultured in the presence of triamine. Results are statistically essential at accepted significance level of 0.01.

Structural polysaccharides, including glucan and chitin, are the greatest group of cell wall constituents. In the case of both resistant strains, the glucan content lower by *ca* 11% in comparison with that in the control strains cultured without triamine was recorded (Fig. 3). Results of variation analysis in Anova test did not show significant differences of chitin content between resistant and control strains. Determined values of F statistic for genus *Aspergillus* were lower than critical value at accepted significance level of 0.05.

The share of lipids in relation to the cell wall mass was 92.5 mg/g d.m. in the sensitive strain of *Aspergillus niger*. Its content increased by *ca* 12% in the resistant strain. An increase in the amount of lipids by 41.3% was noticed, from 78.4 mg/g d.m. to 110.8 mg/g d.m., in the strain of *Aspergillus flavus* cultured in the presence of triamine.

The protein content ranged between 219.2–336.6 mg/g of the cell wall dry mass in the sensitive strains examined. A very slight divergence of protein contend was observed between resistant and control strains of *Aspergillus flavus.* Results differences are not statistically significant. However, increase in the amount of proteins, equal 21.2%, was observed in the resistant strain of *Aspergillus niger* in comparison with the sensitive (control) strain.

The spectra obtained in spectroscopic analysis show the correlation of absorption as a function of the wave number value. No significant spectrum differences between the control sample and the resistant

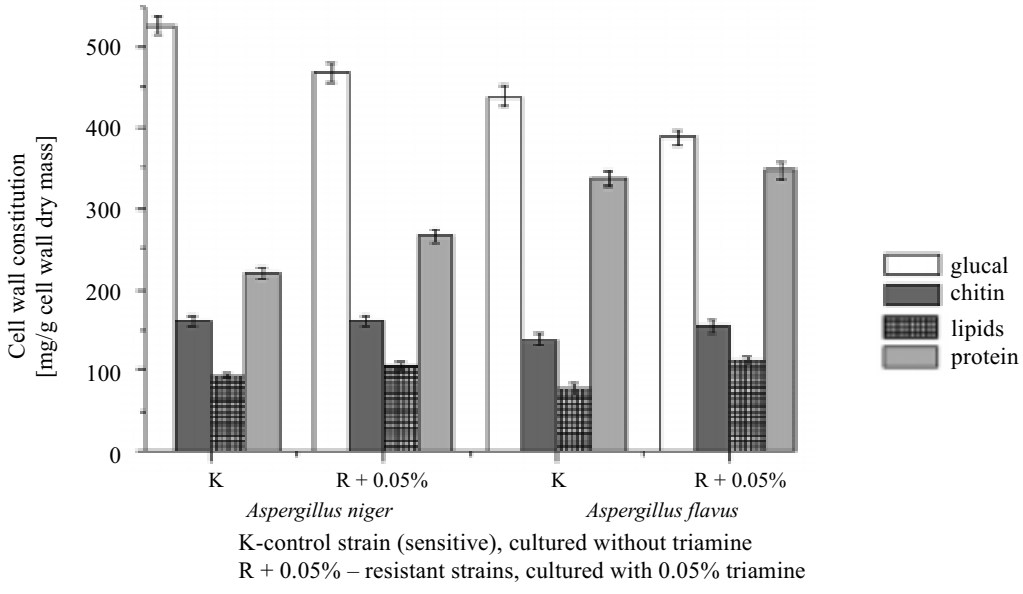

Fig. 3. Cell wall constituents of the sensitive and resistant strains after 7 days of culture

sample were noticed for the cell wall of the strain of *Aspergillus niger* (Fig. 4). Additional absorption bands were not registered; the absorption zone, however, in the range of the wave number 3050–2800 cm^{-1} was slightly stronger for the cell wall of resistant strain. The bands in this range are characteristic of vibrations stretching C-H bonds. Enhanced absorption was also observed in the range 1570–1528 cm^{-1} that corresponds to N-O bonds.

Spectrum differences were observed in the case of the cell wall of the strain of *Aspergillus flavus* (Fig. 5). Most bands disappear on the IR spectra of the resistant strain in comparison with the control sample. Slight peaks are visible below 3000 cm^{-1}, in the range 1700–1800 cm^{-1}, and in the area of 1100 cm^{-1}, which are successively characteristic for vibrations stretching C-H bonds, C = O bonds of carbonyl groups, and C-C bonds.

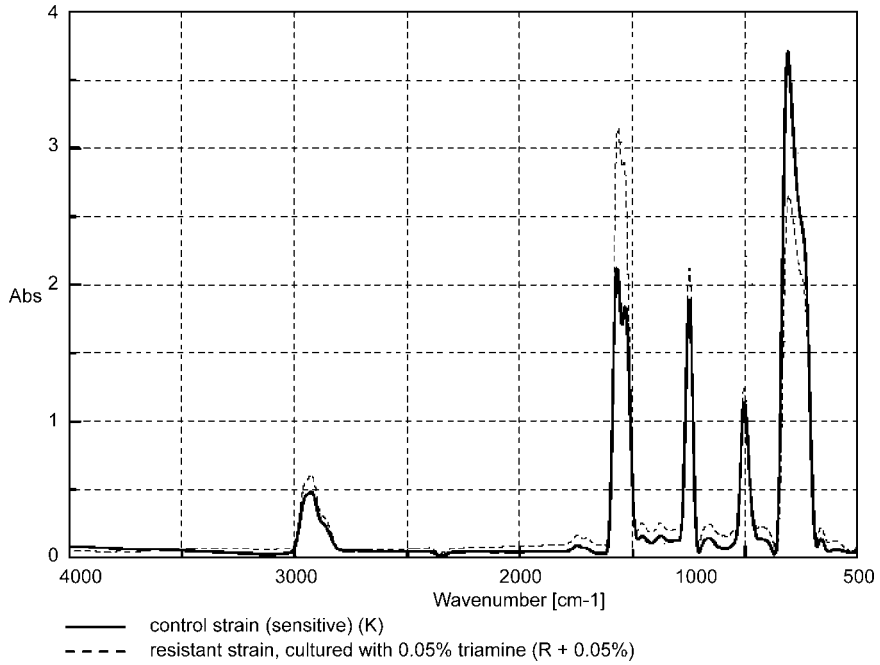

Fig. 4. Spectrum of cell wall of *Aspergillus niger* sensitive and resistant strains

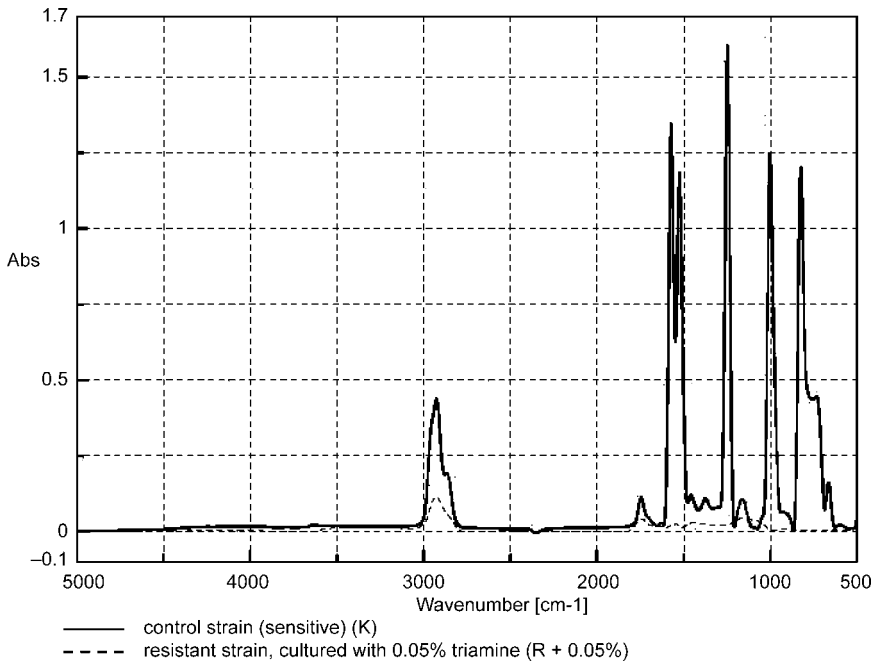

Fig. 5. Spectrum of cell wall of *Aspergillus flavus* sensitive and resistant strains

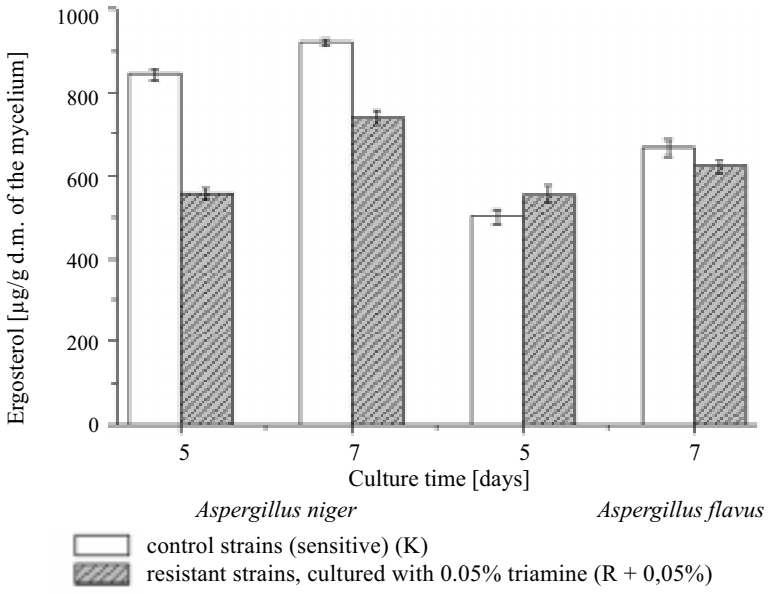

Fig. 6. Ergosterol level in the mycelia of *Aspergillus* strains sensitive and resistant to triamine

The presence of triamine itself, however, in the cell wall was not observed for both strains of the genus *Aspergillus*. The individuals observed on the basis of spectra described above may be products of triamine decomposition or of metabolic processes.

Ergosterol in the mycelium of the sensitive strains and the resistant strains. The examination of obtained data shows that the synthesis of ergosterol in the resistant strain of *Aspergillus niger*, cultured in the presence of 0.05% triamine, was inhibited in comparison with the control (sensitive) strain, not exposed to APDA (Fig. 6).

After 5 days of culture in the presence of APDA, the ergosterol content was lower in the resistant strain by 33.6% in comparison to the control strain. Further growth of this cell membrane constituent was noticed after 48h. The ergosterol content was also higher by 19.65% in the mycelium of the control strain than that of the resistant strain on seventh day.

The sensitive strain of *Aspergillus flavus* was characterised by the ergosterol content lower by 10% than that in the resistant strain after 5 days of culture. Its growth by 161 mg/g d.m. of the mycelium in the control sample and only by 64 µg/g of d.m. of the mycelium in the resistant strain was noticed after 7 days. Because

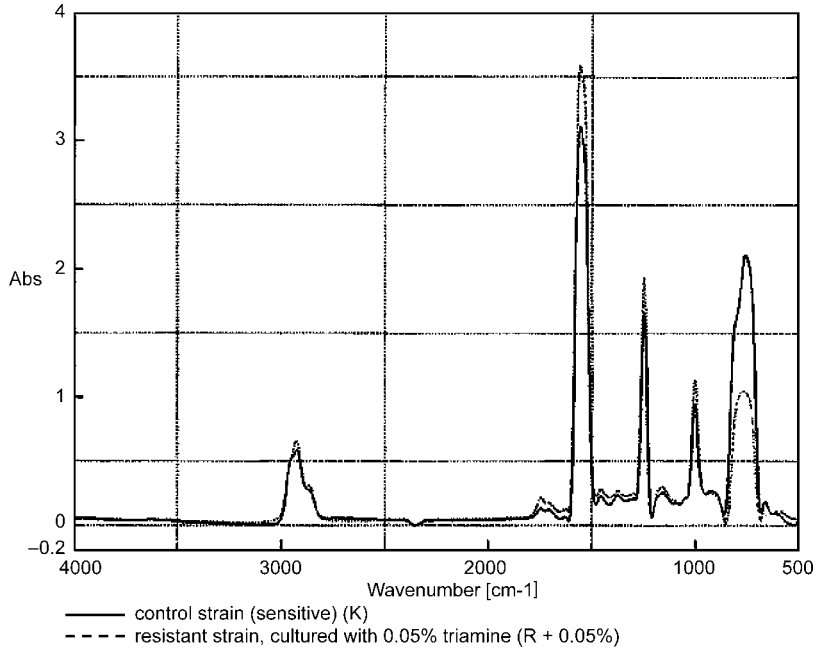

Fig. 7. Spectrum of mycelium of *Aspergillus niger* sensitive and resistant strains

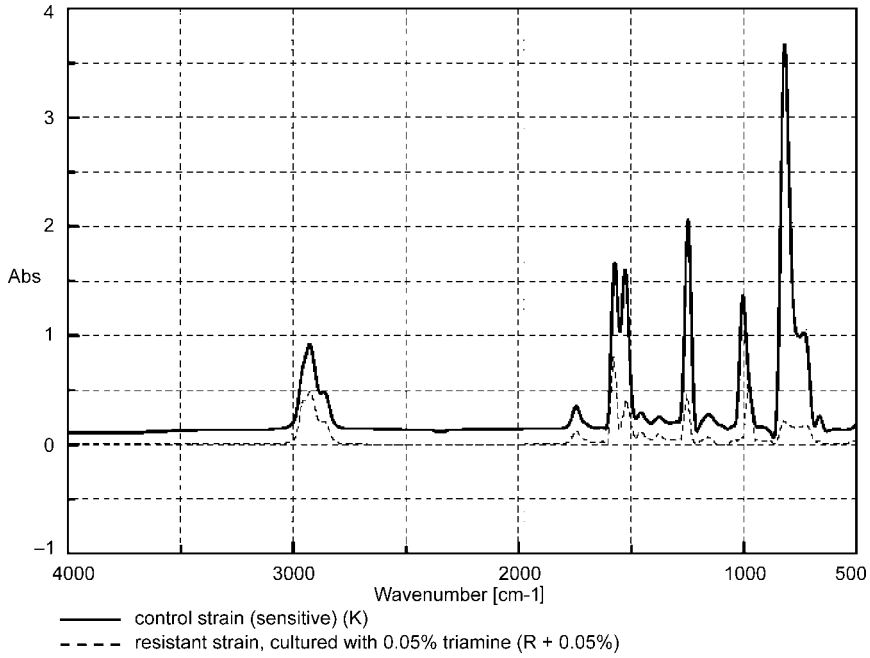

Fig. 8. Spectrum of mycelium of *Aspergillus flavus* sensitive and resistant strains

of such a small increase of tested cell membrane component, a higher ergosterol content was observed for the control strain than for the resistant strain, cultured in the presence of APDA. Differences of ergosterol content between control and cultured mycelium in both growth phases are equal to 6–10%. With the accepted significance level of 0.01, these differences are statistically significant.

Spectrophotometric analysis of the mycelium. To identify the differences between the strains of *Aspergillus* sensitive or resistant to triamine, the dry mass of their mycelia was analysed in the infrared. The examination of the FTIR spectra of the mycelia of both species in the samples with triamine did not show additional peaks in comparison with the control samples (Fig. 7 and 8). This could suggest that no additional functional groups occurred in the mycelia as a result of the presence of APDA. Qualitative changes were not recorded; quantitative changes, however, are noticeable, especially in the wave number ranges 1530–1260 cm^{-1} and 700–800 cm^{-1}. A slightly stronger absorption in the entire spectrum range in comparison with the control sample was noticed in the mycelium of the resistant strain of *Aspergillus niger*. A reverse situation was recorded in the case of *Aspergillus flavus*.

Discussion

The cell wall of moulds belonging to the genus *Aspergillus* constitutes on the average 50% of the mycelium dry mass (Fiema 1994). The cell wall content reached 54% in the resistant mycelia of the genus *Aspergillus*, obtained as a result of ten-fold passages and cultured in the presence of 0.05% triamine. The sensitive strains were characterised by a smaller participation of the cell wall, equal to 46%. The growth dynamics of resistant strains was inhibited.

According to the Bartnicki-Garcia's classification of cell wall structures (Bartnicki-Garcia, 1968), moulds of the genus *Aspergillus* belong to the chitin-glucan category. The content of these structural polysaccharides in the walls of the studied strains is higher in comparison with that given by Rokem *et al.* (1986) and Fiema (1994). As shown by Żakowska *et al.* (1997), the glucan content in the mycelium of sensitive strains of *Aspergillus niger* in the cell wall is 43%, and that of chitin – 12%. These values are higher for resistant strains, and equal to 55% and 20%, respectively. Similar results were also obtained by Kisser *et al.* (1980), who reports the glucan content in moulds cell wall is 60% and chitin content – *ca* 18%. Comparable content values of structural polymers were recorded in the examinations conducted using 0.05% triamine. In contrast to these studies, however, it was noticed that glucan synthesis was suppressed in the cell walls in the resistant strains belonging to the genus *Aspergillus*. The chitin content did not change almost at all.

The resistant strain of *Aspergillus niger*, cultured in the presence of APDA, showed the protein content growth by 21.6%. In the case of *Aspergillus flavus*, an increase in the amount of lipids in the wall of the

resistant strain by 41.3% in comparison with the sensitive strain was recorded. These results contradict those obtained by Żakowska *et al.* (1997), who recorded a lower level of proteins and lipids for strains resistant to various compounds of molasses than that for sensitive ones. Divergent data may be caused by the use of different substrates or the specificity of the examined strains. Lipids constitute spare substances in the cell; they fill up spaces and merge the cell wall. The growth of their content could then be a defence reaction to the exposition to a harmful agent such as N,N-bis(3-aminopropyl)dodecylamine.

Cell wall spectra of the strains examined in the infrared did not reveal the presence of triamine in this part of the cell. The presence of new functional groups that may derive from this chemical compound was not recorded. No qualitative changes were observed in the cell wall of the control and resistant strains of *Aspergillus niger*, and only quantitative changes were noticed. In the case of the resistant strain of *Aspergillus flavus*, the disappearance of most bands in comparison with the control sample was observed. The data obtained in the chemical and spectroscopic analyses of the examined cell walls show, that changes caused by triamine does not occur with the same intensity in both species. These changes probably enhance resistance, and are the defence reaction.

The cell membrane, also containing ergosterol, is another target for antifungal agents in the mycelium. Richardson and Warnock (1995) report that some strains of moulds resistant to amphotericin B were characterised by a reduced ergosterol content. The ergosterol content recorded in the resistant strains of *Aspergillus niger* as well as of *Aspergillus flavus*, subjected to triamine, was also lower than that in the sensitive strains. These findings contradict the data given by Pawiroharsono *et al.* (1987), who reported that an increased ergosterol content in cytoplasmatic membrane protects the cell from unfavourable environmental conditions.

Spectrophotometric analysis in the infrared of the mycelia of both sensitive strains and resistant strains did not reveal significant qualitative differences, only qualitative differences were recorded. The absence of any significant differences was also demonstrated in the analysis of the mycelium ultrastructure (the authors' unpublished studies).

These findings make it possible to claim that the resistant strains belonging to the genus *Aspergillus* adapted to new conditions, unfavourable for them. Their adaptability may be confirmed by the suppressed development of the mycelium of the resistant strains until eighth day, and then its intensification, which may indicate the enzymatic adaptation of the mycelium. The increase in the participation of the cell wall and the quantitative changes that took place in its composition are also indicative of the adaptation to unfavourable conditions.

The results discussed do not comprise all the changes, which reduce the sensitivity to product studied. They confirm, however, the occurrence of moulds resistance in practice. They also indicate types of defence mechanisms developed by growing mycelium as a reaction to a harmful agent.

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