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SECTION I: AGRICULTURAL BIOTECHNOLOGY

ISOPEROXIDASE PROFILES IN SOYBEAN *IN VITRO* CULTURES UNDER OSMOTIC STRESS

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Abstract. *Soybean is commonly used grain legume with multiple product applications. However, its yield is reduced by environmental stress with drought being one of them. Due to the complex nature of drought tolerance, the problem has been studied from different aspects. Biotechnological methods complement classical ones and make possible modeling of stress in vitro. Biochemical analysis of stress related proteins contribute to better evaluation of the tolerant genotypes. The aim of the present work is to compare isozyme profiles of peroxidase in organogeneic soybean cultures cultivated in normal and stress conditions. Adventitious shoots and callus cultures from cv Rosa were induced on modified Fraytag medium. In vitro response of cotyledonary explant was recorded, shoot and callus development in stress conditions was characterized. Clusters of shoots were transferred to control medium and media containing polyethylene glycol (PEG) of concentrations of 4 %, 6%, and 8%. Totally five peroxidase isoforms were identified. Some of them cannot be detected in the control, thus induced under stress conditions. Induction is weaker at PEG concentration of 4% and more pronounced at 6% and 8% PEG. Enzyme profiles in shoots subjected to stress for periods of 1, 3, 6 and 23 days were identified. Differential response was recorded depending on PEG concentration and time treatment.*

Keywords: peroxidase, osmotic stress, soybean, *Glycine max*, *in vitro* cultures

1. INTRODUCTION

Glycine max is considered a world wide strategic crop and commonly used grain legume with multiple product applications. Soybean has a leading position among the legume species as a source of high quality protein and oil and beneficiary valuable substances as isoflavones, phenolic compounds and saponins. (Sakthivelu et al., 2008a) especially recognized by vegetarian and healthy food diets. However, abiotic environmental stresses including drought, high temperatures and salinity reduce yield potential substantially (Boyer, 1982). Due to the great importance of the problem and the complex nature of the tolerance efforts and studies for improvement of plant performance are of different aspects. Classical breeding methods have been complemented with new techniques of plant biotechnology and molecular biology (Bajji et al, 2000, Diab et al., 2008) giving possibility for simulating the desired stress *in vitro,* providing selection on cell level and manipulating genes for resistance.

Plant defensive mechanisms are also in the focus of environmental stress research. A number of stress factors provoke a burst of active oxygen species in plants (Foyer et al., 1994). These free radicals are chemically highly active and cause injuries in plant cell structure. Several enzymes, including peroxidase, are the main defensive mechanism against oxidative stress in plants as they eliminate H_2O_2 and free radicals. Peroxidases (PO) are a large family of enzymes which oxidize different substrates in the presence of H_2O_2, thus regulating its concentration in plant cell. It has been demonstrated that these enzymes are involved in several biochemical and physiological processes including different stress responses (Medina et al., 1999). Increase of peroxidases activity is a general response of plants to stress factors (Fang & Kao, 2000). Effect of water stress (flooding and drought) on antioxidant enzymes have been studied in rice varieties (Mali and Mehta, 1977) and *Anthemis* (Gorjizad et al., 2010)

In Bulgaria soybean breeding have been directed to improvement of yield, biotic and abiotic stress tolerance (Goranova and Todorova, 2005). Recently tissue cultures of soybean have been developed and they were used for ***in vitro*** modeling of abiotic stress and screening for genotypes with higher tolerance (Kosturkova 2005, Kosturkova et al., 2006, 2008, Nedev et al., 2007, Sakthivelu et al., 2008b)

The aim of the present work is to compare the development and the isoenzyme profiles of peroxidase in soybean calli and shoots cultivated ***in vitro*** under normal and stress conditions in attempt to analyze plant response to the drought stress factor.

2. MATERIALS AND METHODS

Preparation of in vitro cultures.

Seeds of soybean cv Rosa were provided by Dr. R. Todorova (patent holder, Todorova and Goranova, 2010) from the Soybean Experimental Station – Pavlikeni. After rinsed with water seeds were surface sterilized by immersion in 70 % ethanol for 1 min, followed by 30 % v/v commercial bleach for 20 min and rinsed three times in sterile distilled water. Seeds were plated under aseptic conditions on basal medium of Murashige and Skoog (1962) for germination. After 10-14 days cotyledonary nodes were excised from the seedlings before the formation of the first leaf and were plated on modified Fraytag's medium (MSF) containing 4 mg/l benzylaminopurine (BAP) and 0.1 mg/l 3-indolebutyric acid (IBA) (Nedev et al., 2007) - normal control conditions. To simulate drought conditions polyethylene glycol (PEG) with molecular weight 6000 was added into the media before autoclaving to have final concentrations of 4 %, 6 % and 8 % w/v. For peroxidase profiling expants responding ***in vitro*** on MSF medium were transferred to media containing PEG. After induction of organogenesis and callogenesis tissues from the green shoots and from the calli were biochemical analyzed on the 1st, 3rd, 6th, 23rd, and 30th day of cultivation in stress (cultured on media containing PEG) and normal control conditions (cultured on media lacking PEG).

Cultivation conditions.

In vitro cultures were maintained in phytotrone rooms at temperature of 25±1°C, photoperiod of 16/8 h and illumination of 40 $molm^{-2}s^{-1}$.

Biochemical analysis.

Calli and shoots from *in vitro* soybean cultures were ground in 0.01 M Tris, 0.08 M glycine, 0.005 M cysteine and 20% sucrose at pH 8.3. Homogenates were centrifuged at 10 000 rpm for 10 min. The supernatant was used as a source of enzyme. Anodally migrating isoforms of peroxidase were resolved on 7.5% polyacrylamide slabs as separating gel with 3% stacking gel according to Davis (1964). The length of the separating gel was 6 cm and stacking gels were 2 cm long. Electrophoresis was conducted at 200 V until the indicator bromphenol reached the gel end. Isoforms were designated by number symbol reflecting their gel migration (in mm) from the start. Staining of gels followed procedureof Przybilska et al. (1982)

3. RESULTS AND DISCUSSIONS

Soybean in vitro cultures development under osmotic stress.

Soybean cotyledonary explants plated on medium for induction of organogenesis started formation of buds from the meristematic tissue (Fig. 1) and gave rise to green shoots for a period of one month. Simultaneously bottom part of the explant was swelling and compact calluses like tissue or callus was formed. This pattern of development of excised cotyledonary nodes was observed for soybean cultures from other varieties investigated previously (Nedev et al., 2007; Sakthivelu et al., 2008b). The negative effect of the osmotic agent PEG was visible at early stage of explant development (second week, Fig. 1) suppressing organogenesis and callus induction. Further formation of shoots and calli and their growth was influenced by the stress conditions in a various way (Table 1). After one month in culture the number of explants that have developed in stress conditions was lower (75 %, 70 % and 60 % for PEG concentrations of 4 %, 6 %, and 8 %, respectively).

However, all *in vitro* responding explants formed shoots and callus simultaneously. Shoot number per explant decreased from 2.3 to 1.1 with the increase of PEG concentration. Shoot growth was arrested too, with exception for 4% PEG. Callus weight decreased significantly (by 30 %) at the lowest PEG concentration of 4 %. However, for the next concentrations of 6 % and 8 % callus growth was stimulated. Higher weight was recorded for the cases of higher stress pressure of 6-8 % of PEG. In addition to the more vigorous formation of callus the latter was more liquidy.

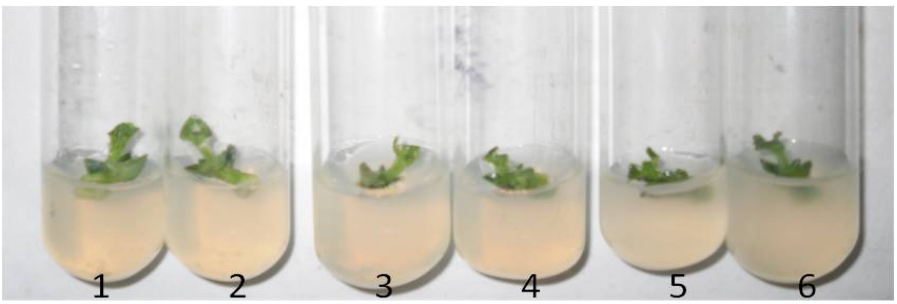

Fig 1. Two weeks old cultures of soybean cotyledonary explants developing on MSF media for induction of organogenesis in normal control conditions (medium without PEG) and under osmotic stress (media containing 4 % and 6 % PEG).

Legend: Concentration of PEG in the media: 0 % PEG, Control in testubes 1 and 2; 4 % PEG in testubes 3 and 4; 6 % PEG in testubes 5 and 6.

Table 1. Characteristic of soybean cv Rosa adventitious shoots and callus cultured of MSF media under normal and osmotic stress conditions on the 30th day of cultivation.

PEG concentration in the media	*In vitro* response [%]	Shoot number per explant	Shoot size [cm]	Callus weight [g]
0 %	85	2.3 ± 0.7	3.2 ± 0.26	0.700 ± 0.27
4 %	75	1.7 ± 0.5	2.0 ± 0.12	0.490 ± 0.14
6 %	70	1.4 ± 0.4	2.8 ± 0.28	0.600 ± 0.15
8 %	60	1.1 ± 0.4	2.1 ± 0.30	0.770 ± 0.18

Values are mean ± SD

Soybean isoperoxidase profiles of in vitro induced shoots and calli.

Isoperoxidase profile of soybean *in vitro* shoots cultivated on different concentrations of PEG for a different period of time is shown in Fig 2. Totally five peroxidase isoforms (10, 21, 24, 28 and 35) were identified. One of them (isoform 10) can not be detected in the control and it is induced under stress. Isoform 10 was less intense on the third day and most intense on the twenty third day. Considering isoform 21, in most cases of PEG treatment its intensity was much greater compared to the control.

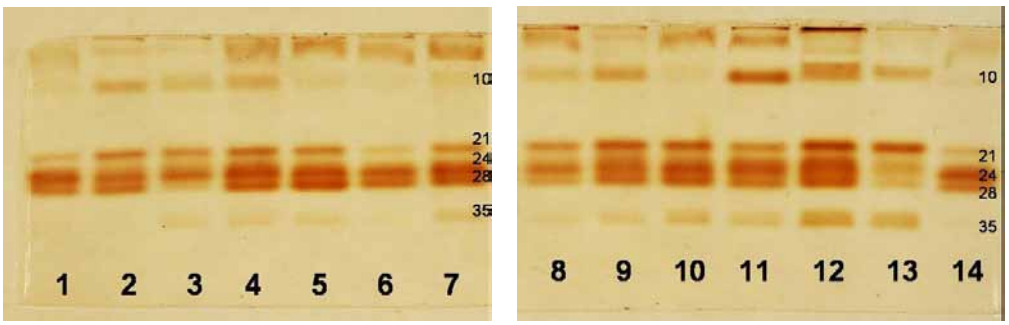

Fig 2. Isoperoxidase profile of soybean *in vitro* shoots cultivated on different concentrations of PEG for a different period of time

Legend: **A**. First day: 1 - 0 % PEG (Control); 2 – 4 % PEG; 3 – 6 % PEG; 4 – 8 % PEG; Third day: 5 – 4 % PEG; 6 – 6 % PEG; 7 – 8 % PEG;

B. Sixth day - 8 – 4 % PEG; 9 – 6 % PEG; 10 – 8 % PEG; Twenty third day: 11 – 4 % PEG; 12 – 6 % PEG; 13 – 8 % PEG; 14 – 0 % PEG (Control)

Generally, there was gradual increase of its intensity parallel to increase of PEG concentration. Isoforms 24 and 28 demonstrated an intensive response to the stress factor. It was most pronounced on the third day (lanes 4-6), sixth day (lanes 9-10) and twenty third day (lanes 11-12). Isoform 35 was absent in the control and in the plants treated with 4%

PEG on the first day. There was increase of its intensity corresponding to the increase of PEG concentration. It was most illustrative on the sixth day (lanes 8-10) and on the twenty third day (lanes 11-13). Isoperoxidase profile of soybean ***in vitro*** cultures treated with different concentrations of PEG for thirty days is presented in Fig 3. Excluding isoform 10, a similar pattern was observed. Isoform 35 was not found in the control. Considering isoforms 21, 24, 28 and 35, the most intense response to the stress factor was recorded in calli cultures (lanes 1-3).

The exposure of soybean shoot cultures to osmotic (drought) stress, simulated by PEG, induced new isoperoxidases as a response to the stress factor. Induction of peroxidase have been observed also in ***Aegilops*** (Landjeva et al., 2004), barley (Tamás et al., 2007) and ***Hevea brasiliensis*** (Dutsadee and Nunta, 2008).

Differential response of soybean peroxidase to the stress factor was recorded depending on PEG concentration and time treatment. Generally, there was gradual increase of isoperoxidase intensity (activity) which corresponded to the increase of PEG concentration. Similar pattern of gradual increase of isoperoxidases was found in ***Aegilops*** (Landjeva et al., 2004) too. Analysis of 13 POD genes in sweetpotato revealed differential expression of PODs in response to the stress factor **(**Yun-Hee Kim et al., 2010).

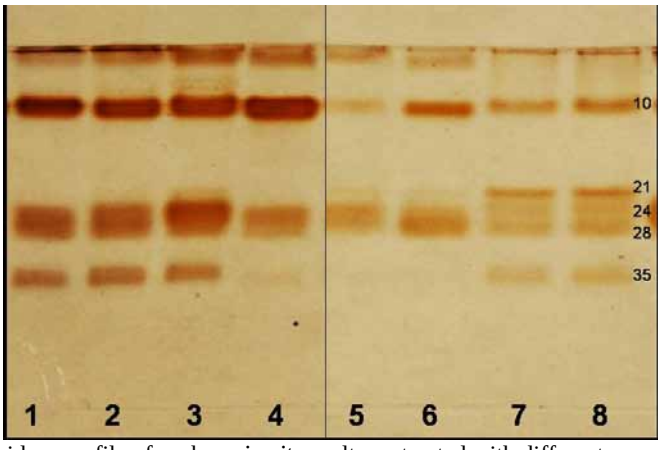

Fig 3. Isoperoxidase profile of soybean ***in vitro*** cultures treated with different concentrations of PEG for 30 days.
Calli: 1 – 4 % PEG; 2 – 6 % PEG; 3 – 8 % PEG; 4 – 0 % PEG (Control);
Shoots: 5 – 0 % PEG (Control); 6 – 4 % PEG; 7 – 6 % PEG; 8 – 8 % PEG

4. CONCLUSION

It could be concluded that induction, increase of activity and differential expression in response to the stress factor are closely linked to an improved antioxidant defense capability mediated by peroxidase in soybean cv Rosa. This variety was chosen in these experiments for its higher yield under non-irrigated field conditions (Todorova and Goranova, 2010) and is considered with higher drought tolerance.

Presented results enrich our previous research on comparison between the response of various soybean lines to water deficiency in field and laboratory conditions using drought simulation by PEG. A tendency of positive correlation between seed yield from one side and seed germination and seedling growth stages from another was observed (Kosturkova et al., 2008). Cotyledonary nodal explants subjected to 6% PEG 6000 treatments, resulted in reduction in shoot induction, compared to the control. *In vitro* callus cultures of both Indian and soybean cultivars grown in Bulgarian showed a reduction in callus growth with PEG treatment, compared with the control. Presence of PEG in the medium elevated dry matter content in all the treatments compared to the control (Saktivelu et al., 2008b).

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AN ANALYSIS OF THE EFFECT OF TEMPERATURE ON THE QUANTITY OF BT TOXIN IN COTTON VARIETIES

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Abstract. *The expression of Bt transgene in cotton cultivars is dependent upon a host of factors. Extreme variations in temperature not only affect overall plant vigour and growth but the expression of Cry1Ac gene is also compromised. The expression of Bt content correlated with temperature was quantified in six cotton varieties during the cotton year 2010-2011. The results indicate a decline in expression of Cry1Ac over temperature independent of the hybrid genotype. The situation necessitates a post commercialization monitoring of the formally approved cotton varieties.*

Keywords: Cry toxin; quantity; temperature; correlation

1. INTRODUCTION

Bt cotton was developed to thwart the bollworm's attack and during the last couple of years, there has been a steady growth in area covered by Bt cotton varieties in Punjab (figure 1).If on one hand, there is an increase in the adoption of Bt technology, there are also many Bt varieties of unknown parent germplasm.

The expression of Bt toxin is dependent upon a host of factors including parent germplasm, plant parts, availability of macronutrients especially nitrogen and changes in temperature. According to the work done in India, the expression of toxin in boll-rind, squares and in the ovary of flowers was found below the threshold of 1.8µg/g ,the amount considered as LD^{99} (Kranthi et al., 2005).

A high dose expression of Bt toxin ensures the durability of Bt technology and it is important that the foliar plant parts express this endotoxin in sufficient quantities to cause maximum mortality of the target pests i.e. *Helicoverpa armigera* (Hubner), *Earias vitella* (Fabricius) and *Pectinophora gossypiella*.The first instar of these target pests completes development on leaves chewing the tender foliar tissues. An enhanced quantity of Bt endotoxin, therefore, in leaves is desirable in domestically developed and commercialized Bt cotton varieties.

However, the temperature extremes affect the plant vigour leading to a decrease in production of Bt endotoxin. The expression of Cry1Ac in Bollards due to temperature variations have been found varying and correlated with the survival of target pests (Olsen et al., 2005).The present study was designed in order to verify the uniformity of optimum expression of Bt toxin with temperature variations in commonly commercialized cotton varieties during the cotton season 2010-2011. It was necessary because earlier research has shown a variance of Bt toxin in illegal varieties commonly found in farmers' fields.

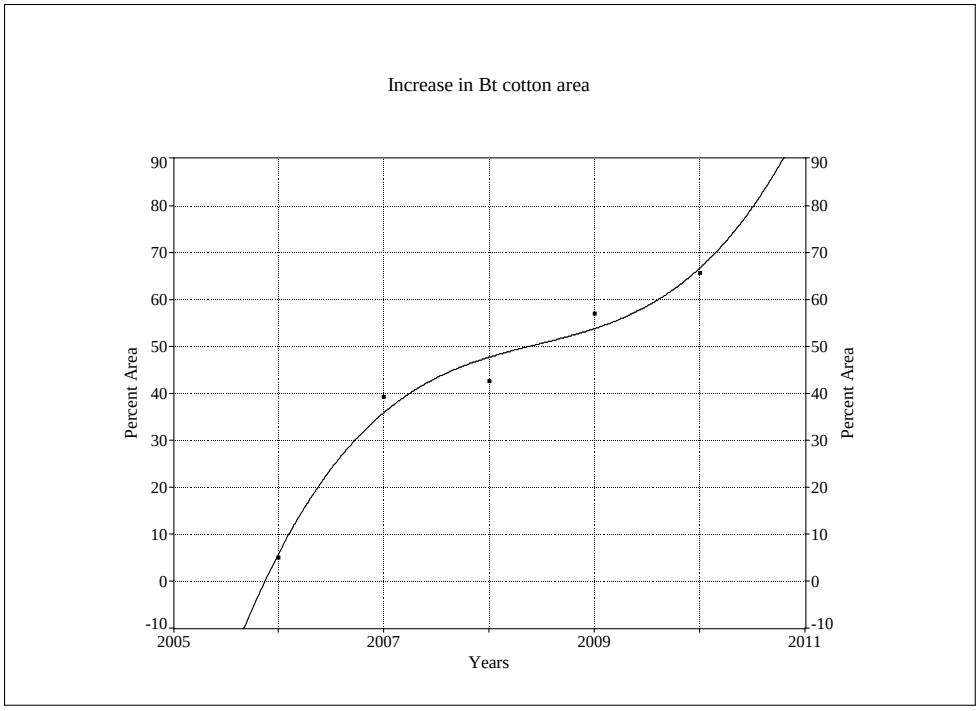

Figure 1: Evolution of Bt cotton area in Punjab 2006−2010

2. MATERIALS AND METHODS

Nine cultivars of *Gossypium Hirsutum* L. containing cry1Ac gene (MON531 event) were grown in RCBD design. The varieties were Ali Akbar 802, IR 3701, Ali Akbar 703,FH 113,IR 1524,GH 2035,FH 114,N 121 and MG 6. The plot size was 40 x 20 ft keeping row to row distance 30 inches and plant to plant distance 12 inches. The sowing date was May 15, 2010.Water and fertilizer were applied as per normal recommendations.

Three consecutive plants were selected from each genotype in every repeat and tagged after 30 days of germination. Selected plants were tested for their Bt studies. Agdia immunostrips were used for Bt testing. Cry1Ac protein concentration was quantified in cotton leaves by ELISA. After 30 days of sowing, leaf samples were collected and preserved in liquid nitrogen for ELISA test. Third leaf from the top of each tagged plant was tested. Tagged plants were quantified five times i.e. 30 days after planting (DAP), 70DAP, 100DAP, 130DAP and 160 DAP.

Average temperature of days since sowing/previous sampling date was calculated prior to each sampling. Bt quantification data was analysed by split plot design by using QI Macros 2011 software which showed a non normal data nature. At α=0.05, the data rejects null hypothesis.

3. RESULTS AND DISCUSSION

The quantities of Bt toxin determined over the growing season are expressed below (table 1).

Table 1: Variation in the quantity of Bt toxin over the growing season

Mean Temperature	37^0C	39^0C	36^0C	31^0C	26^0C
Varieties	30DAP	70 DAP	100 DAP	130 DAP	160 DAP
AA802	0.921	0.755	0.672	0.546	0.541
MG6	0.915	0.764	0.722	0.548	0.514
IR3701	0.924	0.76	0.681	0.546	0.523
AA703	0.92	0.749	0.716	0.548	0.521
FH113	0.924	0.765	0.727	0.546	0.527
IR1524	0.913	0.756	0.657	0.549	0.506
GH2035	0.912	0.765	0.673	0.543	0.504
FH114	0.928	0.744	0.636	0.544	0.519
N121	0.924	0.743	0.634	0.548	0.516

The trend of change in concentration of Bt toxin related with temperature shows the highest productivity at 37^0C which declines onwards with an increase in temperature (figure2).
The Bt toxin concentration is expressed in µg/g.Bt toxin concentration ranged between 0.930—0.510 µg/g. The maximum level of Bt protein was produced at 30DAP while minimum being at 160 DAP. The concentration of Bt content is tapered off towards the end of the growing period.
At 37^0C, i.e. at 30DAP, the varieties produced the highest quantity of toxin.The juvenile tissues produced the maximum effect.However, at this stage the bollworms do not infest the field as they appear later in the crop season at about 80-100 DAP. At 26 ^{0}C (160DAP), the plants had the favorable temperature for growth and mature plants produced Bt toxin sufficiently. However, the graph clearly indicates that at no growth stage, no plant variety at any temperature range produced Bt toxin high enough to cause 95% mortality of target pests (high dose strategy).Illegal varieties produced by random crossing of Bollard varieties with local high yielding cultivars not only cause an economic loss to farmers (as the Bt seeds are priced higher than non Bt); but also cause the survival of target pest individuals. The resistance evolution case has not yet been observed in Pakistani cotton fields as no research report indicates so. Pakistani cotton experts are of the view that the resistance evolution in target pests due to less-than-optimal toxin concentration is not feasible (Sharma, 2009). The farmers have small holdings and due to adjacent fields of different crop plants, target pests do not survive due to host unavailability in vicinity. However, in many cotton areas especially nearby cities, there is a trend of vegetable growing nearby cotton fields. The migrating surviving pests may find ochra, brinjal and cauliflower as alternate hosts for development.

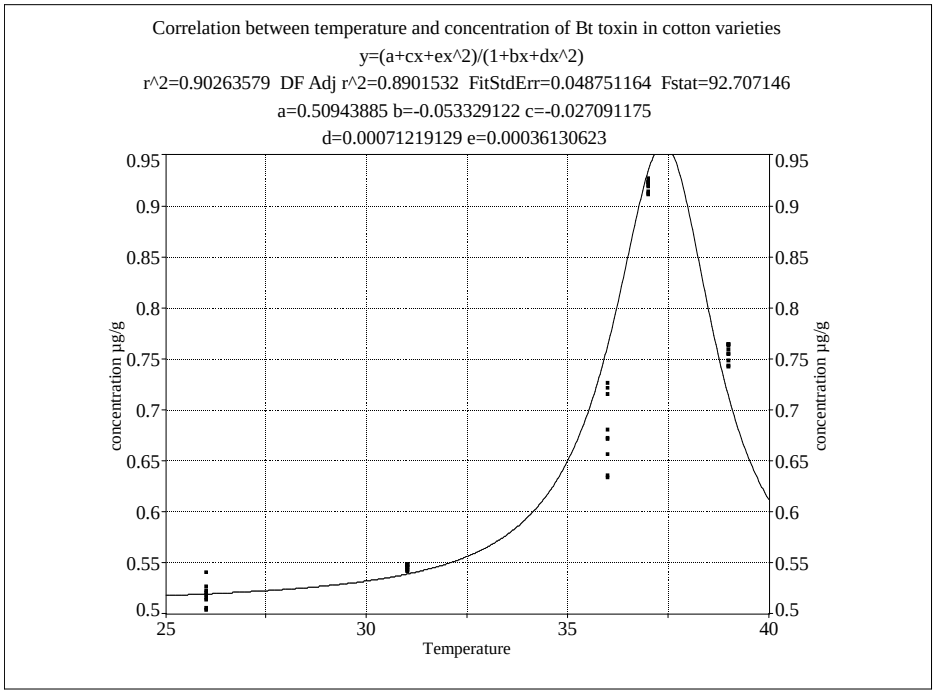

Figure 2: Variation in concentration of Cry toxin with temperature

4. CONCLUSIONS

There is an imminent threat of resistance evolution in target cotton pests if proper resistance management measures are not adopted.The study indicates the need for provision of certified Bt seed accessible to all farmers. A strict regulatory regime must be established to monitor and to contain the spread of illegal varieties as well as the environmental re-evaluation of commercialized varieties for their Bt potential. It is necessary because in pre-release trials, the assessments are limited in scope and the assessment results may differ in different climatic conditions and agronomic practices by the farmers.

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A MODEL POST COMMERCIALIZATION MONITORING FRAMEWORK FOR BT COTTON IN PAKISTAN

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Abstract. *Cotton varieties were formally approved for large scale cultivation in Pakistan in late 2010.However, the prevalence of Bt cotton varieties in farmers' fields can be traced back since 2002.The cultivation of unapproved varieties with an unknown parent germplasm and a doubtful compatibility of such varieties with local flora must be analysed for environmental consequences. The flow of transgene in local ecosystem needs to be monitored at earliest. A regulatory model is designed for the monitoring of ill effects/spread of Bt transgene. The model presents a plan from sampling to results interpretation involving the active role of all agricultural stake holders in Pakistan.*

KEYWORDS: Bt cotton; stakeholders; monitoring; framework

1. INTRODUCTION

When GM crops are approved for unconfined environmental release in Pakistan, systematic post commercialization monitoring (PCM) of their environmental impact is not required. In Pakistan, GM crops are produced by conventional breeding, mutagenesis or more commonly by recombinant DNA techniques. In 2010, Pakistan was among the top 5 countries producing Bt cotton (James 2011).Viewed retrospectically, GM hybrids changed the farming practice in cotton fields by allowing a reduction of spraying frequency and related practices.

Although not legally required to conduct PCM, Pakistan needs to establish the PCM regime after the pervasiveness of illegal cultivation of GM cotton. As the GM crop is commercialized on a larger spatial and temporal scale, it may be possible to observe smaller and less frequent ecological risks (Snow et al., 2005).Thus PCM provides the basis for an early warning system to detect and mitigate any adverse effects.

2. MATERIALS AND METHODS

A review of existing monitoring legislations and the role of regulatory authority in Pakistan are studied. A model Monitoring plan in post commercialization scenario is developed.

ENVIRONMENTAL RISK ASSESSMENT OF GM CROPS

Over a couple of years, technology developers have partnered with researchers from provincial and federal institutions to conduct monitoring of specific environmental and agronomic concerns previously identified in the Biosafety Guidelines 2005.An appropriate risk assessment gathers data that clearly link the measurement endpoints with the environmental management goals through the assessment end points (Raybould, 2006).

PROJECT MANAGEMENT AND MONITORING PROCEDURE

Once the environmental risk (assessment endpoints and testable hypothesis) are characterized, the next phase is project management and monitoring. A sound governance structure is essential for successful PCM. A principal investigator would coordinate planning and execution of the study. It would be prudent to have national and international advisors to provide expert advice on all aspects of the PCM program.

a) THE MONITOR

Pakistan has a long history of conducting field and farmer surveys by public researchers or extension workers. However the same level of cooperation between farmers and employees of the technology developers may not be as forthcoming because of perceptions of bias or trust.

In Pakistan, neither the regulator i.e. National Agriculture Research Council (NARC) nor the technology developer multinational companies has the expertise or infrastructure to conduct PCM. Much more importantly real or perceived conflicts of interests should be avoided by having public academic lead PCM, not the developer with a vested interest in the commercial success of the GM crop or the regulators that approved the unconfined environmental release of a GM crop; because there is the risk of bias in validating their original decision.

Public academia should have access to these protocols directly from NARC as a condition of the regulatory process for a genetically modified crop (GMC). Without these molecular protocols it would be impossible to confirm the presence of a transgene of interest in GM crop populations.

b) THE FINANCER

Cost sharing should reflect the value of the information to private developers, the public good and the policy development. Technology developers should bear the majority of costs because they gain the major financial gains through seed or pesticide sales. A 20 years patent on the technology ensures a monopoly for that duration and an acceptable rate of return on investment. Moreover results from the PCM provide technology developers with new information to help farmers adopt best management of GMCs and optimize economic benefits.

c) SPATIAL SCALE

Depending on the size of the risk being monitored or regional scale of GM crop cultivation, multiple monitoring sites with different eco-regions will be required to determine the variation in magnitude of measured variables. Data often would be collected in cultivated fields, field borders margins, and/or adjacent ruderal or natural areas for areas adjacent to GMC cultivation, pollen flow and seed dispersal distances will influence the spatial scale of monitoring.

d) TEMPORAL SCALE

The duration of PCM should generally be 5 years from time of market release and wide spread cultivation of a GM crop (Kjellsson and Strandberg, 2001).The responsibility of

possible longer term (5 yrs) PCM to detect potential interactive or cumulative GM crop

specific effects (e.g. invasiveness) should lie with a reporting network of trained observers. Existing personnel in agriculture (extension specialists), environment and natural resource management could constitute a reporting network after receiving training in recognizing special ecological effects of GM crops.

e) DATA COLLECTION

A plethora of data will be collected from PCM. Quality control is essential in data entry, storage compilation and analysis. A common template for standardized data entry using common or comparable database software will facilitate efficient data compilation and management. Graef et al., (2007) stressed data harmonization and coordination among all stakeholders for ensuring data quality and comparability. Given the significant costs in collecting PCM data, it is imperative that proper checks and balances are in place for professional data base management.

f) DATA ANALYSIS

Both univariate and multivariate statistics are employed for data analysis. Because data variability is inheritability greater in the field or landscape scale experiments than small plot research, the type I error rate in univariate statistical analysis is generally greater than 0.05—typically 0.10-0.20.A statistician specialized in analyzing large data sets from field/landscape research is an integral part of the PCM team.

g) DATA EVALUATION

After the data are statistically analysed a final report will be written and scientific publications submitted for peer review. Technology developers should not be allowed to restrict or impose conditions in legally binding agreements on contents of reports or peer reviewed publications. The full results must be made available in a timely manner to the scientific community and society at large. A transparent system is essential for allaying concerns that any perceived environmental risks are being trivialized.

THE REGULATORY DECISION

The results of the data analysis to confirm or refute the hypothesis will dictate the decision by National Biosafety Committee (NBC). Once a decision is made it must be conveyed to all stakeholders particularly farmers via means of mass communication. Once the PCM of a GMC is terminated, a cost benefit analysis conducted by the third party may improve the efficiency and effectiveness of future PCM activities. A model monitoring Plan for the Environmental management of commercially approved GM crops in Pakistan is presented below (figure 1).

Sites for the GMC invasiveness would be established in all crop growing areas mostly in Punjab and Sindh where GMC and non GMC crops are mostly adopted by the farmers. For each site, monitoring will terminate at the end of study period. If the effect size was exceeds at a site; monitoring may continue for two more years. The life span of the crop may influence the temporal scale of monitoring. In addition, the adoption rate of the GM crop may also influence the duration of monitoring. Here a model monitoring plan is designed for GM cotton in Punjab (table 1).

The selection of baseline for comparative risk assessment is important. In case of cotton crop in Pakistan, as the pest control measures are of practical relevance so the consumption rate of pesticides in transgenic versus non transgenic crop cultures can make a baseline as a risk assessment factor.

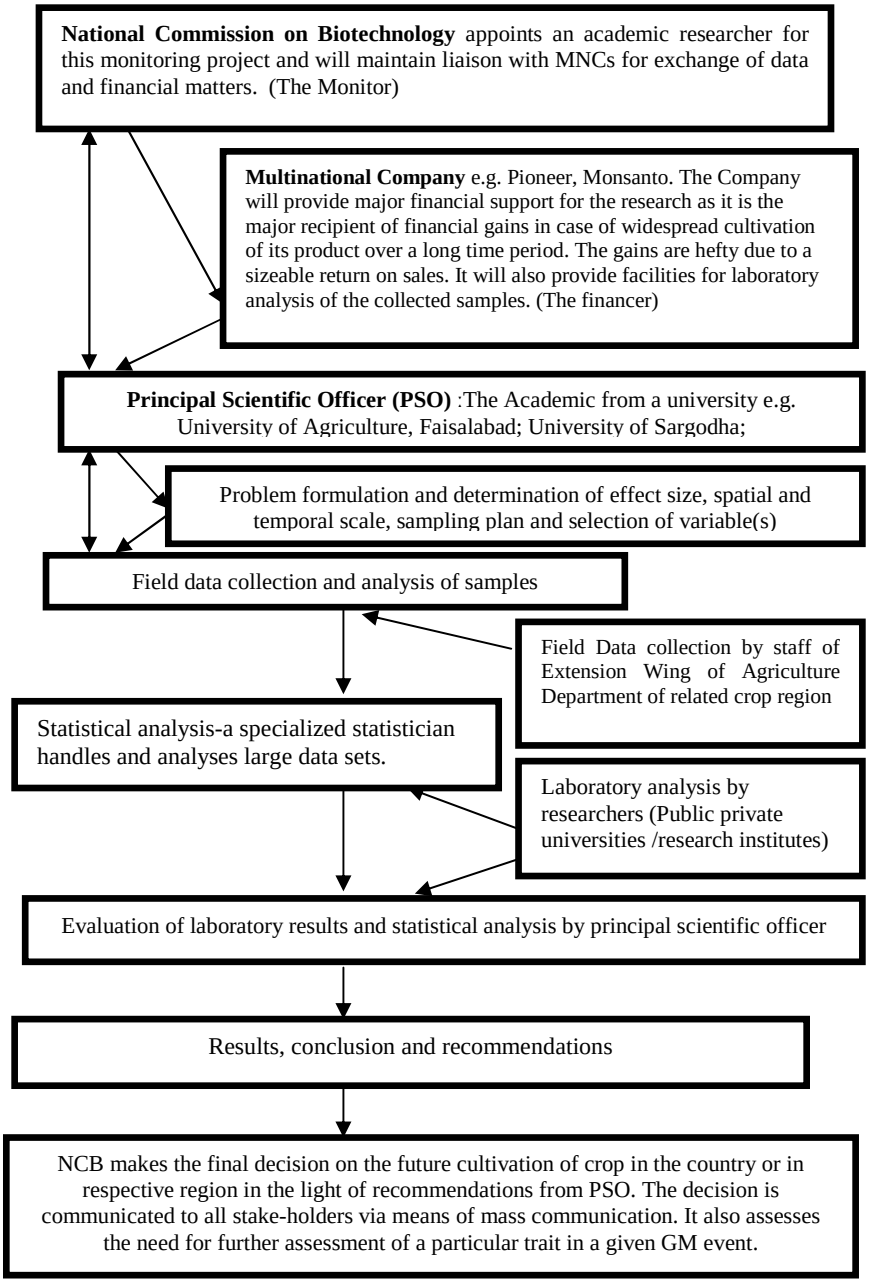

Figure 1: A model representation of post commercialization monitoring system for GM crops in Pakistan

Table 1. Post commercialization monitoring system for ERA of GM cotton in Pakistan

No.	Step	Description
1	Risk Identification	◆ Increased resistance in targeted lepidopterans
2	Assessment Endpoints	◆ Comparison of resistant individuals' i.e. lepidopterous larvae feeding on plant parts of IR cotton.
3	Effect Definition	◆ Hypothesis: IR cotton provides LD^{99} against target insect species.
4	Effect size	◆ Minimum and maximum effect will range from 0.1% to 10 % of the population size of the crop plants in case of ferals.
5	Spatial Scale	◆ Cotton growing belt of Punjab especially Southern Punjab
6	Temporal Scale	▪ An optimum size study consists of the minimum number of assessment end points at a given time to make the study economical and time saving. The study will focus on one assessment end point (max.2) at a time and will commence after three years of approval for commercial scale cultivation of the GM crop to let the GM crop plants and their effects assimilate into the local environment in a variety of growing areas. ▪ However, in case of illegal/unapproved cultivation in pockets of crop area, the study may begin earlier due to the factors of varied quantity and quality of toxin production. At least two crop seasons must go through rigorous assessment.
7	Sampling Plan	❖ The principal scientific officer will contemplate the sampling scheme with consultation of the statistician to arrange the variables and border line of the data to be collected. ❖ 50 sites of GM crop and 50 sites of non-GM cultivation are selected (on an average) in a province for two years. GM and its comparator will bear the data for 300 crop years collectively at the end of data collection period. ❖ Composite index of insect population size in each habitat—fled margins, ruderal areas, undisturbed grassland area. ❖ For insect collection, the number of insects on crop plants per $1m^2$ ring after every 5 meters distance will be counted in a representative 1 ha area per one evaluation site.
8	Variable Selection	◆ For insects, the variable is the comparative number of insects per 1 ha. area in both crop types.
9	Data collection	◆ Weather data for each site compiled from the closes meteorological station ◆ Ferals: minimum of 25 1-m^2 quadrate measurements in each of field. ◆ For insects data is collected on weekly basis throughout the growing season till maturity.
10	Data Analysis	◆ Analysis of variance using a mixed model, with site and year considered as random effects and treatments as a fixed effect (significance level of $P \leq 0.10$ for insects. ◆ Repeated measures analysis to detect any changes over time. ◆ Means comparison test (t test) to determine if effect size exceeded. ◆ Data analyzed within farming system by district and combined data set.
11	Data Evaluation	◆ Determine the frequency of susceptible and resistant target and non target insects in the vicinity.
12	Regulatory Decision	◆ Environmental risk assessment is validated if null hypothesis accepted (effect size never exceeded).Buffer zones are setup in districts where the null hypothesis is rejected (effect size exceeded; expand mitigation measure if effect size is increased into two or more regions over the study period.
13	Communication	◆ Decision by the NCB will be communicated to farmers and the public in general in conjunction with the study participants.

4. CONCLUSIONS

For the ecological management of the released GM event,there is no official mechanism for systematic post commercial monitoring of GM crops.It is possible that the unwanted flow of transgenes is detected in large scale agriculture that went undetected during preapproval field trials because the trials are of relatively small scale and have short duration.There are sufficient assessment criteria for new GM crops before their commercialization.But,becasue uncertainties exist,it is useful to establish some probalistic framework and compare expected environmental benefits and costs.

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INVESTIGATION OF NEW AGRICULTURAL DECISIONS FOR THE MAINTENANCE OF THE SOIL FERTILITY OF THE VERTISOIL

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Abstract. *The changes in the soil, climatic and economic conditions lead to the application of new effective decisions for the agricultural activities to full use utilization of the productive potential of the cultivated cultures. The new production technologies contribute to the maintenance of the soil fertility.*
In the report are treated the results of three years examination of technologies for polish production in two crop–rotation.
It was found out in the investigation that with the applying of new decisions for the soil cultivation and mainly for the mineral fertilization the increase of the crop from 13.4 to 17.0% of the cultivated cultures could be achieved. A tendency towards improvement of the values of some of the main physical and agrochemical parameters of the soil fertility is established.

Keywords: soil tillage, fertilization, soil fertility

1. INTRODUCTION

The problem with technology actualization in farming and more specifically in grain producing is current and socially significant. Alternative technological solutions meeting the effectiveness criteria – increase of productivity, decrease of investment, rational use of resources and keeping the soil fertility – are a prerequisite for successful business.

The main solutions for achieving economic stability in grain producing through maintenance of soil fertility concern soil treatment as a direct agro technical measure and as a factor, affecting the effectiveness of other farming practices, the main one of which is fertilization, and also the means of fighting weeds vegetation. The rationalization of main agro technical measures allows for fuller use of the reproductive potential of crops grown at a good level of soil fertility.

The purpose of this research is to examine new solutions for application of main agro technical means – soil treatment, mineral fertilization and the weed control, with which a better upkeep of soil fertility in leached vertisoil is achieved.

2. MATERIAL AND METHODS

This research has been carried out in a field experiment set in Bozhurishte testing facility, Sofia District. The scheme of this experiment includes two crop rotations with the alternation between winter crops and row crops in the first rotation and fused surface crops in the second. Three agro technical measures have been studied:

Soil treatment – three soil treatment systems are included in Table 1, the new solutions being: system 1 – line loosening at a depth of 15-18cm when row crops are grown; the second system compares options with simultaneous plowing and harrow tillage, as well as sowing and rolling, with such, where these operations are conducted independently.

Table 1. Soil treatment systems

№	Crops	year	Treatment systems		
			O_1	O_2	O_3
1.	Triticale Wheat	2008-2009	Loosening 12-15cm	Plowing 15-18 cm	Disk plowing 10-12 cm
2.	Coriander Corn	2009-2010	Plowing 28-30 cm	Plowing 28-30 cm	Loosening 28-30 cm
3.	Wheat	2010-2011	Disk plowing 10-12 cm	Disk plowing 10-12 cm	Disk plowing 10-12 cm
4.	Beans Rape	2011-2012	Loosening 20-25 cm	Plowing 20-25 cm	Loosening 20-25 cm

Fertilization – non-fertilized options T0 and agro chemically tested for fertilization rate soil samples have been compared. At an equal rate of fertilization the following options were tested: T1/ fertilization with ammonium nitrate, superphosphate and potassium sulphate; T2/ fertilization with ammonium nitrate, foliar fertilizer with 30% nitrogen, 1/5 of the quantity needed for feeding up, amophos and potassium sulphate. The potassium fertilizer is introduced during the first crop rotation in the first year for wheat and during the second in the second year for corn.

Weed control – includes options with integrated control, using different chemical substances.

The soil of the testing area is leached vertisol, a typical representative of the heavy kind – physical clay content 78-80% and il content 62%. Soil is medium humus 3.5, characterized by high bufferity and plasticity. Despite of that, signs of overdensification of the under-plowing layer are present.

3. RESULTS AND DISCUSSIONS

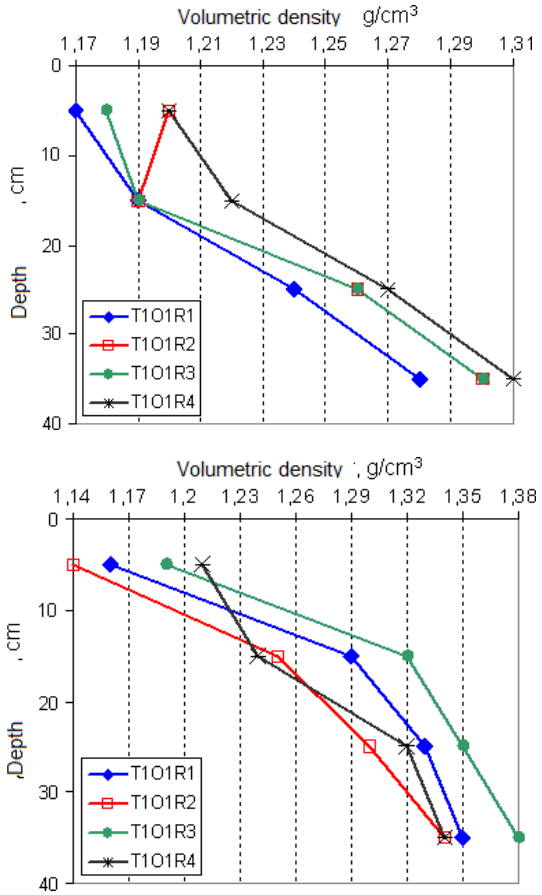
Soil fertility is identified with the conditions of nutrition of crops. Some physical factors which affect the development of the root system also play an important role. Solutions in soil treatment mainly affect the structural condition, the water supply, the volumetric density and the firmness of the soil.

The structural analysis of the soil shows that mostly in dry sieving the aggregates are of a size > 1 mm. The structural aggregates are waterproof but with a very firm composition. With depth increase, the fraction of the smallest aggregates decreases in dry as well as wet sieving. After water saturation about a third of the aggregates sized > 1mm are left in the 30-40 cm layer.

The content of soil moisture is a main physical parameter, which defines the dynamics of processes taking place in the soil, the living conditions for beneficial

organisms in it, as well as germination, growth and development of crops grown. No influence on the soil moisture level has been registered by the applied treatments.

The comparative analysis of data shows that when combining the plowing and the harrow tillage and the sowing with rolling, the pressure on soil's top layer is decrease, which expresses itself in the decrease of volumetric density by 0.02-0.035 g/cm^3, and of the firmness by 8,5 to 11, 7 kg/cm^2. The reported differences by options of treatment are sharper in the corn test area in the 10-20 cm layer – up to 0,13 g/cm^3, which is due to the compaction action of the wheels of aggregates during pre-sowing treatment. Examination of main physical indicators in corn shows that conducting of intercrops loosening helps keeping their values within optimum. Figures 1 and 2 show the values of volumetric density, which in the option with intercrops loosening for plowing layer 0-30cm are lower by 0.023 g/cm^3 in comparison to the cultivation plots.



Figures 1, 2. Volumetric density of soil in g/cm^3 when growing corn

The firmness of soil before ear formation of wheat decreases its values by an average of 20 kg/cm^2, while the plots with Triticale keep theirs in the same range. The option with disk plowing of wheat reads higher values for layers 10-20cm and 30-40cm, while the plot with Triticale shows higher firmness of the options with plowing – up to 100,80 kg/cm^2. Firmness of soil has values that are not critical for root system development of the crops grown during the second year. Before coriander flowering, the values of this soil parameter increase with an average of 15 kg/cm^2, while the plots with corn, the soil keeps its firmness within the same range.

The total porosity of the vertisols has values that are lower than these, established during optimum condition of the soil. In the shallow layers of the soil this decrease is mainly at the expense of coarse pores with equivalent diameter >200μ. The share of fine pores increases, as well as the difficult to access water, contained in them.

From the analysis of the main parameters defining the physical characteristics of soil diversity – leached vertisol, one can establish that moisture, volumetric density and firmness have values that is good from the agro technical point of view and does not limit the development and functions of plants' root system.

The agro chemical analysis found that the mineral nitrogen content is low, with a variation of data present in the respective samples. Although oat was sown as an equalization crops, no complete equalization of the area's agro chemical parameters was achieved, due to the many years of experimental options applied to the fertilization levels. The content of mobile forms of nitrogen, which in the output samples was 3,0-7,4 mg/kg soil for the test area of the first and respectively 3,9-5,5 mg/kg soil for the area of the second crop rotation, as a result of the mineral fertilization in the phase of ear formation of the crops is with a higher level. With wheat in the 0-20 cm layer for the fertilization options a mineral nitrogen content of 9,7-12,9 mg/kg soil is registered, and with Triticale - 13,2-19,7 mg/kg soil. In the 20-40 cm layer these values for nitrogen are lower. There is no registered concord of data for options only fertilized with ammonium nitrate and those fertilized with liquid fertilizer in the tillering and spindling phases. Higher content of the area with Triticale is explained by the lesser number of plants per unit area and accordingly weaker absorption by plants. Data analysis shows a slight tendency of increasing of assimilable phosphorous when fertilizing with amophos compared to fertilizing with triple superphosphate. From the analysis of soil samples taken during ear formation of the crops, we have established an increase of soluble potassium by 3-6 mg/100g soil in the options fertilized with potassium sulphate.

With corn in the 0-20 cm layer a content of mineral nitrogen of 10,3-16,1 mg/kg soil was read, and with coriander 13,9-22,4 mg/kg soil respectively. In the 20-40 cm layer the values of nitrogen are lower. For corn options fertilized only with ammonium nitrate and those fertilized with foliar fertilizer in phases 5 leafs and 9-10 leafs. Higher content of the area with Triticale is explained by the lesser number of plants per unit area and accordingly weaker absorption by plants. Data analysis shows a slight tendency of increasing of assimilable phosphorous when fertilizing with amophos compared to fertilizing with triple superphosphate. Potassium content is satisfactory and for the plowing layer it's in the 26.0 – 38.0 mg/100g soil boundaries. From the analysis of soil samples taken during gathering of the crops, we have established an increase of soluble potassium in the options fertilized with potassium sulphate.

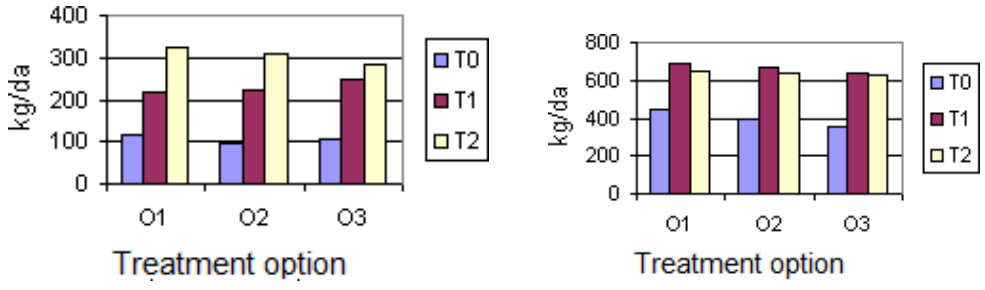
A cumulative expression of the effect of applied production technology is the quantity of production. Relatively low yield has been gathered in the fertilized options but compared to the average for the region they are higher, especially with wheat. Figure 2 shows that compared to the non-fertilized options (in the continuation of 20 years), the fertilized plots have provided a satisfactory yield. Results from dispersion analysis for wheat show that the treatment system has no statistically significant impact on yields. The only statistically proven fact is the effect of fertilization (at a level of probability p<0,01%), it being 85,16% of the total data variation. Highest average yield is gathered in option T2, using amophos as a phosphorous fertilizer and the usage of nitrogen foliar fertilizer (proven at NMDR 0,1%). These results are correct for the concrete climatic conditions during the experiment year.

For Triticale crops again the only proven effect is the one from fertilization (at a level of probability p<0,1%), it being 84,99% from total data variation. Highest yield for the specific climatic conditions during the experiment year were gathered in fertilization option T1 (using amophos as a phosphorous fertilizer and the usage of nitrogen foliar fertilizer). The usage of traditional soil mineral fertilizers gives a lower rate of productivity.

Fertilized options gave a relatively high yield of corn. Figure 3 shows the yield from different options. It is obvious that compared to the unfertilized options (in the continuation of 20 years), the fertilized plots have provided a satisfactory yield. Results from dispersion analysis of data show that the treatment system has statistically significant impact on yields at a level of probability p<1%. The biggest statistically proven effect is that of fertilizing (at a level of probability p<0,01%), it being 90,90% of the total variation of data. With the three treatment systems (O1, O2 and O3) there is a proof of differences in yields between the two fertilized options and the non-fertilized (a proof rate of 0,1%).

As a result of the loosening, conditions for fuller usage of the biological potential of corn have been created in mid-lines, manifested in an increase of grain yield in options T0O1, T1O1 and T2O1 with 24,0 kg/da, 40,7 kg/da and 22,5 kg/da respectively (Fig. 4). The combination of appropriate treatment and fertilization is most effective in option T1O1. If the correct tool is used (Paraplow) with which this loosening affects also the zone under the main root mass, the effect will be significant.

For coriander crops the tendency is similar to that with corn. The usage of traditional soil mineral fertilizers gives a lower level of productivity, with differences between the two options for fertilization (T1 and T2) are proven at p<1%.



Figures 3, 4. Yields from wheat and corn in kg/da

Directions, which can be sought for efficient production, are mainly related to the economizing of production and streamlining of costs.

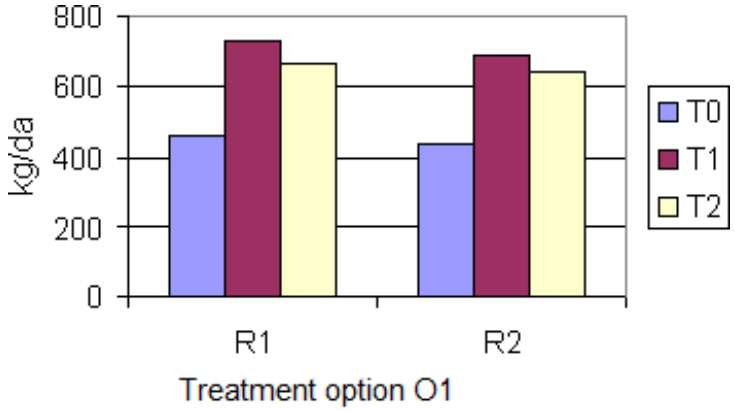


Figure 5. Corn yields depending on the mid-line treatment

4. CONCLUSIONS

This experiment determined that the application of new solutions of soil treatment and mainly of mineral fertilization, one can achieve an increase of yield of 13.4 to 17.0% of crops grown. The exchange of part of the ammonium nitrate, intended for nutrition with a double introduction of foliar fertilizer, as well as the usage of amophos as a phosphate fertilizer, increase the productiveness of crops grown on leached vertisol.

A tendency of improvement of the values of some of the main physical and agro chemical parameters of soil fertility has been found.

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TECHNOLOGICAL EVALUATION OF THE USED SIDERATES WITH AN ALTERNATIVE BIOTECHNOLOGICAL METHOD OF RE-CULTIVATION

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Abstract. *The alternative biotechnological method is based on the usage of green fertilization as a source for accumulation of organic mass, needed for the process of humification. With it, however, the construction of correct crop rotations is accompanied by many problems. The main requirement is that more modest cultures are grown, developing in poor and unstructured soils with a poor water and air regime (2, 3, 4, 5.) The number of treatments is reduced to the possible limit so that the processes of densification and denitrification are limited. This, on its part, strongly narrows the species composition of the cultures (1, 4.)*
The purpose of this research is to reach a quick accumulation of organic matter in the newly-formed substrates and appropriate soil conditions for the growing of siderate cultures in a compacted succession of crops.

Keywords: re-cultivation, siderates, biomass, organic matter

1. INTRODUCTION

The alternative biotechnological method is based on the usage of green fertilization as a source for accumulation of organic mass, needed for the process of humification. With it, however, the construction of correct crop rotations is accompanied by many problems. The main requirement is that more modest cultures are grown, developing in poor and unstructured soils with a poor water and air regime (2, 3, 4, 5.) The number of treatments is reduced to the possible limit so that the processes of densification and denitrification are limited. This, on its part, strongly narrows the species composition of the cultures (1, 4.)

2. MATERIAL AND METHODS

In a field experiment on an area of 1 ha in a heap Mednikarovo in the area of mines Maritsa-East, four soil substrates were tested by application of non-humus re-cultivation technology:

1. Sediments of yellow-green and grey-green clays;
2. Sediments + 700 m^3/ha soil from humus horizon on leached vertisol;
3. Sediments + 700 m^3/ha soil from humus horizon + 500 m^3/ha ash;
4. Sediments + 500 m^3/ha ash.

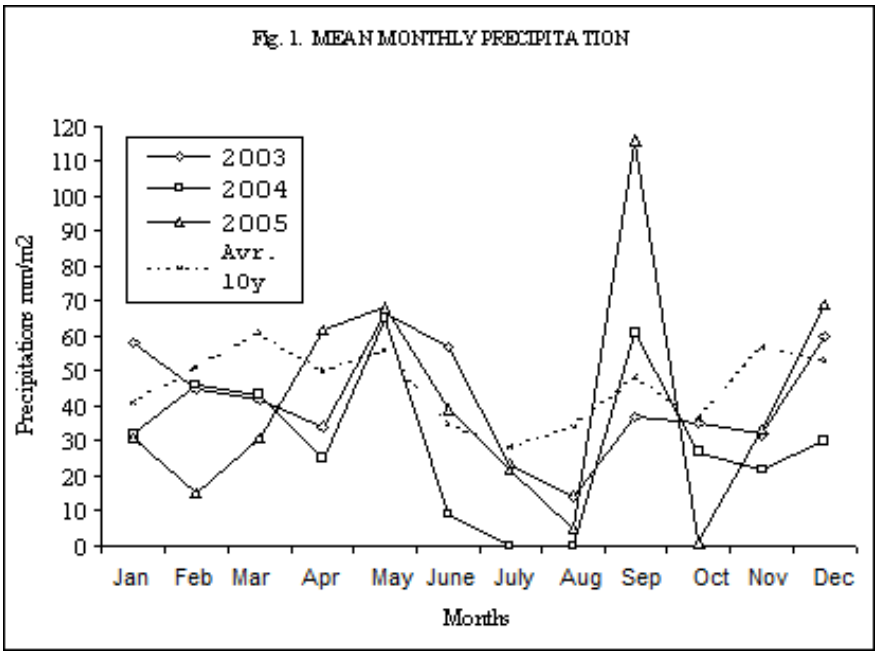
In a compacted three-field succession of crops: I year – winter forage pea + Sudan grass; II year – winter pea + barley (mix) + Sudan grass; III year – wheat, the following treatments were applied (Table 1):

Table 1. SOIL TILLAGE SYSTEMS

Crops	Technology operations		
	Autumn	Pre sowing	Complementary
1. Winter peas 2002/2003	1.Plowing 23-25 cm Sept.-2002 г	Disk plowing 8-10 cm Disk plowing 6-8 cm	Disk plowing 10-12 cm (sole)
2. Sudan grass 2003	2.Plowing 16-18 cm May- 2003 г	Cultivation 6-8 cm Disk plowing 6-8 cm	Rolling after sowing
3. Peas + barley 2003/2004 г	3. Plowing 20-22 cm Sept. 2004 г	Disk plowing 6-8 cm twice	Rolling after sowing
4. Sudan grass 2004 г	4. Plowing 16-18 cm May 2004	Cultivating 6-8 cm twice	Rolling after sowing
5. Wheat 2004/2005 г	5.Plowing 18-20 cm July 2005	Disk plowing 10-12 cm Disk plowing 6-8 cm	Rolling after sowing

The amount of crop and root residues from green fertilization is defined using the meters method, accordingly 1 m^2 and 0.25 m^2.

The air temperature does not deviate substantially from average from previous years. The amount of rainfall is described in Fig. 1. From the data one can conclude that the winter crops are guaranteed enough water from rainfall, and the vegetation of Sudan weed goes with a substantial sole moisture deficit.



3. RESULTS AND DISCUSSION

The implementation of the adopted soil tillage system met serious difficulties from the very beginning. During the autumn of 2002 because of poor content of moisture in the soil and the big compacting of area / 1.75 – 1.90 g/cm^3 / as a result of meliorative works and embankment of substrates, the necessary depth of tillage could not be reached and therefore, neither could the homogeneous mixing of the components. With its meliorative role, the winter forage pea showed extremely high technological qualities and adaptabaility, given the relative unfavorable soil conditions. Other than the not-so-good pre-sowing preparation of the soil and the low content of organic matter, another limitary factor was the high density of the sowing layer, because of which the roots of the pea were placed mainly in the 0-20 cm layer, and under that depth only separate roots. The total quantity of the received by the soil absolutely dry biomass /Table 2/ is significant, especially in option 3, where it reaches 5650 kg/ha. The bio productivity of peas, grown as a separate culture is best expressed in option 3 with component humus soil material and ash. Its analog, option 2, in which there is no ash, has 16% less bio mass. Despite of the found lodging of the crop, the plant debris were very well minced and distributed across the test area, using the rotor silage harvester – KIR – 1.5.

Table 2. BIOMAS CONTRIBUTION TO THE SOILS
(aboveground residues + root residues abs. dray mass kg/ha)

Years	Crops	Treatments			
		1	2	3	4
2003	Peas	3190	4770	5650	3310
	Sudan grass	15400	10740	10910	8310
2004	Peas barley mix	2790	5510	7670	4540
	Sudan grass	6930	8030	7070	4070
Total		**28310**	**29050**	**31300**	**20230**

Peas F=14.34 F crit=4.06 p=0.001 Sudan grass F=44.94 Fcrit=4.06 p=0.00002

For the growing of the winter peas + barley mix, a quality pre-sowing preparation of the soil and even sowing were carried out. The climate conditions were very suitable and an optimum density and height of 80cm were reached in the options with addition of humus soil to the substrates. The received quantity of biomass is highly dependable on the type of soil substrate. It is almost three times lower in option 1 compared to option 3. The improved soil conditions – drainage and aeration in option 3 have given the winter peas and barley the opportunity to show their full productive potential.

The results from two years of growing Sudan grass show that it is a very appropriate crop as a source of humus formation, given the xerophytic properties of the area. During the first year the main limiting factor for productivity was the prolonged drought period. As a result the crop was under-trimmed, the yield being a function only of the number of plants - R^2 =+0.86. This shows that wherever the crop was trimmed enough, the reserve of spring moisture in the soil was used and therefore the yield is highest Option 1(Table 2). During the second year of growing Sudan grass, a significantly lower yield of biomass was received, because of poor soil preparation due to worsened weather conditions. Unlike the peas, the Sudan grass adapts better to substrates not containing ash, which could be explained by the clay soils' ability to hold the scarce soil moisture during

the crop's vegetation for longer periods of time. The biomass yield from Opt. 1 exceeds by 24.2% and 80.4% received from Options 3 and 4.

The wheat yield from the third year of the crops succession is the end assessment of the technological aptitude of the siderate crops grown. The actual yield and the accounted biological yield of wheat are shown in Table 3. Despite the fact that Sudan grass is known to be an inappropriate predecessor of wheat, during this experiment the wheat yield was above 4000 кg/ha. This speaks of a significant effectiveness of the technology applied during biological re-cultivation. From this fact we can derive that the adverse effects of the predecessor can be overcome by keeping the C:N ration lower than 25 by adequate mineral fertilization.

Table 3. Wheat yield 2005

Treatments	Biologic yield		Real yield
	g/m^2	kg/ha	kg/ha
1	434.3	4340	4000
2	475.3	4750	4170
3	491.7	4920	4250
4	436.3	4360	3670

4. CONCLUSIONS

The full technological evaluation of the aptitude of grown as siderates crops, gives the grounds to consider that growing winter forage peas and Sudan grass in a compacted crops rotation is absolutely successful in the humus-free biological method of re-cultivation. Crops show their biological potential very well and give high biomass yields. By growing siderate crops, a fast pace of organic matter accumulation in the soil is reached and this guarantees the successful development of following crops. Except for the specific climatic conditions during crops vegetation, another very important factor is observation of agro technical requirements in production. An omission of any of the needed agro technical operations is a significant risk to the crops growing.

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STUDY OF THE SEED MICRO FLORA IN WHEAT AND BARLEY GROWN IN ENVIRONMENTALLY FRIENDLY CONDITIONS

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Abstract. *By the method of placement in Chapek environment, 40 samples of wheat and barley grown in environmentally friendly and conventional agriculture conditions are set.*
The total contamination of samples has been determined and fungi have been isolated in clean environment and have been defined by species. The mycological analysis showed that the following species of fungi are found: Fusarium, Alternaria, Mucor, Penicillium, Helminthosporium, Cladosporium,Botrytis and Rhizopus. In the makeup of the seminal micro flora, the percentage of fungi species Fusarium (53%) and Alternaria (32%) is significantly higher compared to that of the other species.
The average percent of contamination in seeds is higher in these samples than in cultures grown conventially. Seeds from the conventional field have stronger Fusarium fungi infection, and the materials from the ecological field have stronger Alemaria infection.

Keywords: wheat, barley, fungi, micro flora, seeds

1. INTRODUCTION

Problem in ecologically clean foods and feed production is current on a global scale. Technology for conventional agricultural production used by now, leading to a high increase of productivity is connected to a high degree of pollution, disruption of natural functions and ecosystem integrity, and above all leads to the loss of enormous quantities of non-renewable energy (Knauer.N.1993;Spedding,C.R.W.,1988).
Methods of plant protection had the biggest impact on ecosystems, primarily – the chemical method, which led to mobilization of genetic volatility of harmful species and increased the effect of the pesticide "boomerang" (Stancheva, 1999;).
As an answer to these negative processes in agronomy, alternative systems of agricultural production were created and developed. Bulgaria, as a part of Europe, is a part of these problems. 90% of the area in the country are suitable for the production of ecological foods and by 2013 a sharp increase of areas for biological production is expected.
Cereal cultures are of the most important species that are being grown biologically.
Their growing in ecologically friendly conditions requires the development of a technology, combining stable methods of production (crop rotations, mechanical treatment, sustaining of soil fertility by fertilization with manure and plowing of crop residues, biological control of pests).
Benign and healthy seeds are needed for sustainable yields and quality crops of cereal foods and feed, as a great part of the seed transmitted pathogens can provoke rotting, decrease in germination, and in later stages – systematic infections that can destroy big parts of the crops (Bateman, GL and H. Kwasna. 1999., Chong, LM and JE Sheridan. 1982., Glazek, M. 1997., Kubiak K. and M. Korbas.1999.).

In conventional production, seed pathogens are controlled by fungicides but in bio-farming no effective methods of control are defined. This requires the study of seed pathology in different ways of cultivating the crop in order to establish methods of biological control against phytopathogenic seed parasites.

2. MATERIAL AND METHODS

This experiment has been carried out in the Plant Protection Laboratory of Agricultural Institute in Shumen.

Test subjects were wheat and barley seeds harvested in 2006, 2007, 2008 and 2009 from the institute's experimental fields, where two six-pole crop rotation fields were set up with cereal and grain legumes, grown by two different technologies – eco-friendly and conventional.

The eco-field was fertilized only with manure and no pesticides during vegetation were applied. If needed, the application of harmless for the useful micro flora and entomofauna medicines is allowed, included in the so called "Green list".

With cultures grown by conventional technology, the field is fertilized with mineral fertilizers and pesticide treatment is used against sicknesses and vermins.

Those two fields were created on an identical soil type with similar parameters for nutrient reserves. Soil in testing fields is carbonate black soil with high humus content in the plowing layer (3,7 – 3,74%) and weak alkali reaction (pH 7,7 – 8,1).

Materials for phytopathological analysis of crops grown in eco-friendly conditions and by conventional technology were included in the tests.

Seed analysis was carried out by the method of placement in Chapek environment, 100 seeds for phytopathological evaluation being set from all four options (wheat and barley from the eco-field and wheat and barley from the conventional field).

After a 7 days cultivation period in a thermostat at t 24-26^{0}C pure cultures have been isolated from the test plates from the conventional methods and the pathogens in samples have been defined.

The count of infected seeds for different species of fungi has been reported in % and the statistic processing has been carried out by the Mudra (1958) method.

3. RESULTS AND DISCUSSIONS

After the mycological analysis it was determined that microscopic fungi of the following 8 species are present in tested samples –Fusarium, Alternaria, Mucor, Penicillium, Botrytis, Cladosporium, Rhizopus and Helminthosporium.

Fig. 1 shows the average percentage of contamination of seeds with the according fungi.

It stands out that in the composition of seed micro-flora, the percentage of Fusarium fungi (53%) and Alternaria fungi (32%) is significantly higher than that of other fungi (Mucor, Penicillium, Botrytis, Cladosporium,Rhizopus and Helminthosporium), whose percent of contamination varies in a narrow range (1%-6%). Similar results are reported by other authors studying the seed pathology in wheat and barley (Valcheva, A., 2006; Karadjova, I., 1979; Ivanov, M., 1980), who find highest percentage fungal infection with speciesFusarium and Alternaria.

Fig. 2 shows the ratio of different kinds of Fusarium species in samples. The highest total percentage is that of seeds contaminated with F.graminearum (50%), followed by F.moniliforme (36%) and F.oxysporum (14%) which shows that among fungi of Fusarium species on barley and wheat grown in North-East Bulgaria, most common are F.graminearum.These results differ from Valcheva's (studied fusariosis on those same plants in North-West Bulgaria), where for the period 1994-2004 she has found a greater percentage of contamination with F.moniliforme.

Table 1 shows the mycological status and the total percentage of infected wheat and barley seeds from the eco- and the conventional fields during the period 2006-2009.

Data shows that fungiAlternaria and Fusarium are noted in different quantities in all samples from both crops in separate years. Most infected with Fusarium fungi are materials harvested from the conventional field (average wheat – 34%, and barley – 16,5%). Seeds infected by Alternaria can be found in larger quantities in the eco-field (average wheat – 18,5% and barley – 16,5%).

The presence of the so called "storage fungi" in the complex of seed parasites (Mucor and Penicillium) is weak (average 0,5 – 3%) and no significant differences are found in separate crops, as well as the same crops, cultivated in different conditions.

Microscopic fungi from the species Botrytis are detected only in wheat samples from the eco-field, and species Cladosporium and Rhizopus are isolated only in barley samples, grown by conventional technology.

Seed infection with pathogens from species Helminthosporium is registered in very small quantities (1,5 – 1,75%) in analyzed materials from the conventional field.

Table 2 shows data from statistical processing and compares results from the phytopathological analysis of both crops – wheat and barley grown by the eco- and conventional technologies regarding the total contamination of their seeds with fungi from the species Alternaria and Fusarium during the four-year period of the study.

Analysis shows that there is a proven difference in resistance against seed pathogens from the species Fusarium of wheat and barley grown in eco-conditions.

With conventionally grown barley, fungi from the species Alternaria have a proven stronger participation in samples, and in eco-wheat this difference is very well insured.

4. CONCLUSIONS

- The percentage of fungi of the species Fusarium (53%) and Alternaria (32%) in seed micro-flora is significantly higher than that of other fungi (Mucor, Penicillium, Botrytis, Cladosporium, Rhizopus and Helminthosporium), whose percentage of contamination varies in a narrow range (1%-6%).
- From fungi of the species Fusarium the highest percentage of infected seeds is that with F.graminearum (50%), followed by F.moniliforme (36%) and F.oxysporum (14%).
- Fungi of the species Fusarium and Alternaria are found in all tested samples.
- Seeds from the conventional field are more contaminated with fungi Fusarium, while the eco-field is more contaminated with fungi Alternaria.

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EFFECT OF DIRECT ELECTRIC CURRENT ON VIRUS ELIMINATION IN GRAPEVINE

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Abstract. *The meristem culture and thermotherapy are individually or in combination the most frequent methods of obtaining virus-free plants. The application of heat treatment for virus-elimination in plants is time and energy consuming and needs special equipments (chambers for heat treatment). In the case of the meristem culture, the percent of virus elimination is influenced by the genotype, the type of the virus and especially of the difficulty of meristem excision (the percent of virus elimination is inverse proportion to the explants dimensions).*
The use of electrotherapy with discontinuous electric current followed by in vitro culture was investigated as alternative technique to eliminate grapevine viruses, in simple or mixed infections at a time, in seven Vitis vinifera L. varieties naturally infected. The study had in view the most dangerous viruses of this crop: fanleaf virus (GFLV), arabis mosaic virus (ArMV), leafroll associated virus 1 (GLRaV-1), leafroll associated virus 3 (GLRaV-3), and fleck virus (GFkV). Direct electric current of 1000 and 10 000 kHz was applied for 10 min at the cut ends of one budded herbaceous cuttings. Treated axillary buds were grown on M&S basic medium containing growth regulators. The efficacy of virus elimination process was assessed by enzyme-linked immunosorbent assay (ELISA) testing of regenerated acclimated grapevine plants. The analysis showed some encouraging results for elongated viruses GLRaV-1 and GLRaV-3 in single infections and no virus eradication in mixed infections had been obtained. The electrotherapy of GLRaV-1- infected material produced maximum 12.5% virus-free plants at 1000 kHz and 4.5% GLRaV-3-free vines at 10 000 kHz for 10 min of exposure. The result regarding GLRaV-1 elimination was not reproducible in the case of mixed infection containing it. No satisfactory results have been achieved for isometric viruses eradication (GFLV, GFkV) either in simple or mixed infections.

Keywords: ***Vitis*, virus, eradication, electrotherapy, discontinuos electric current**

1. INTRODUCTION

The International Council for the Study of Virus and Virus-like Diseases of the Grapevine (ICVG), recognizes over 70 infectious agents affecting grapevine (viruses, viroids and mycoplasmas). Many of them cause disorders that reduce the plant vigour and longevity or the quality and quantity of the yield. Infected propagating material is largely responsible for the spread of these diseases among and within viticultural regions. Thus, all efforts should be made to improve its sanitary conditions and to protect selected healthy clones from new infections.

The presence of fanleaf virus (GFLV), arabis mosaic virus (ArMV), leafroll associated virus 1 (GLRaV-1), leafroll associated virus 3 (GLRaV-3), and fleck virus (GFkV in simple and mixed infections can cause severe damages to infected grapevine

plants, therefore the use virus-free vines for multiplication and new vineyard establishing is required (F. MANNINI [1]). In cases of high incidence of infectious diseases in breeding vineyards and impossibility to select authentic and disease-free material of a particular variety or rootstock, several techniques are being applied for virus elimination (I. TSVETKOV & al. [2]).

The meristem culture and thermotherapy are individually or in combination the most frequent methods of obtaining virus-free grapevines (G. GRAMMATIKAKI & A. AVGELIS [3]; B. KŘIŽAN & al. [4]) The application of heat treatment for virus-elimination in plants is time and energy consuming and needs special equipments (chambers for heat treatment). In the case of the meristem culture, the percent of virus elimination is influenced by the genotype, the type of the virus and especially of the difficulty of meristem excision.

Virus-elimination techniques such as: chemotherapy (A. PANATTONI & al. [5]), electrotherapy in continuous electric field (I.C. GUȚĂ & al. [6]), cryotherapy (SH. BAYATI & al. [7]), somatic embryogenesis (G. GAMBINO & al. [8]) and their combinations are considered alternative method to thermotherapy. Each of the listed method involves one stage of *in vitro* culture.

Is well known the use of electric current in the cleaning of viral diseases in plants; instead, a deep knowledge of this phenomenon theoretical basis is not yet available. The experiments demonstrated that the cleaning process nature is, essentially, the effect of the viral protein denaturation, by means of the heat to which they were exposed in the thermal bath that the vegetal tissue constitutes (J.E GONZÁLES & al. [9]).

Electrotherapy has been applied successfully to eradicate potato virus X from different infected potato clones (H.F. LOZOYA-SALDAÑA & al. [10]); dasheen mosaic virus in malanga (J. IGARZA CASTRO & al. [11]; banana streak virus in banana (P.R. HERNÁNDEZ & al. [12]).

The electrotherapy for GFLV elimination was investigated, as a more practical alternative technique comparatively to heat therapy of virus infected grapevine, but not satisfactory results have been obtained. However, the decreasing of by enzyme-linked immunosorbent assay (ELISA) values in function of the exposure period have been registered (J.G. BURGER [13]).

The electrotherapy followed by *in vitro* culture of shoot apices produced 33.33–66.66% GLRaV-1+3 –free plants, regardless explant position of exposed grapevine in continuous electric field (I.C. GUȚĂ & al. [14]).

The aim of the present research was to eliminate fanleaf virus (GFLV), arabis mosaic virus (ArMV), leafroll associated virus 1 (GLRaV-1), leafroll associated virus 3

(GLRaV-3), and fleck virus (GFkV) in simple or mixed infections at a time, in seven *Vitis vinifera* L. varieties, by electrotherapy with direct electric current.

2. MATERIALS AND METHODS

***Source of virus-infected material*:** The study regarding GFLV, ArMV, GLRaV-1, GLRaV-3, GFkV and their combinations (virus complex) elimination has been done on seven *V. vinifera* L. cultivars naturally infected (Table 1), maintained in the grapevine virus infected collection of NRDIBH Ştefăneşti–Argeş. One year old infected plants obtained from one bud cuttings, confirmed as virus-infected by ELISA testing, constituted the source of nodal fragments (microshoots).

Table 1. Virus-infected grapevine cultivars used in the experiment

Grapevine cultivar	**Virus/virus complex**
Fetească albă	GFLV
Fetească neagră	GLRaV-1
Frâncuşă	GLRaV-1
Cabernet Sauvignon	GLRaV-3
Grasă de Cotnari	GFkV
Unidentified genotype (1)	ArMV + GFkV
Unidentified genotype (2)	GFLV + GLRaV-1

Electrotherapy: In the case of electrotherapy with direct electric current, a source that converts the alternating current from electricity network in alternating current with adjustable parameter is required. The experiment consists of subjecting a nodal fragment to an alternating current for a certain period, after that it is inoculated on culture medium, being the source of regeneration of new grapevine plants.

The experimental parameter were established after previous attempts (direct electric current of 1; 100; 1000 and 10 000 kHz was applied for 5; 10 and 20 min at the cut ends of one budded herbaceous cuttings), regarding the viability of microshoots exposed to the action of electric current (unpublished data). The current frequency (1000 kHz; 10 000 kHz) and exposure time (10 min) for this experiment were established. Thus, the experience is bifactorial of subdivided parcels type, 2x7 experimental variants; for each variant were exposed three microshoots as repetitions.

The control for each variant was represented by virus infected microshoots *in vitro* cultured unexposed to direct electric curent (untreated). The position of the nodal fragments on the shoots were identically for all experimental variants, namely the second internode below the apex.

In vitro culture: Nodal fragments subjected to electrical current treatment and virus- infected untreated nodat fragments (control) have been inoculated on M&S (1962) basic medium (T. MURASHIGE & F. SKOOG [15]) containing 2 µmol L^{-1} benzylaminopurine (BAP) and 5 µmol L^{-1} indolilacetic acid (AIA). After three subcultures

of 30 days each, when a multiplication process occurred, individualized and elongated microshoots of 2-2.2 cm were cultured on rooting medium (E. VIŞOIU [16]). During the regenerative processes, the parameters such as multiplication rate (X) and number of percent of rooted microshoots on experimental variants were monitored.

Virus elimination assessment: In order to assess the virus elimination efficiency, regenerated acclimated vines were analyzed by ELISA. The ELISA test was performed according to the method described by M.F. CLARK & A.N. ADAMS [17] with commercial reagents produced by BIOREBA, Switzerland. Absorbance (OD_{405nm}) was recorded by PR 3100 photometer. For a correct discrimination of negative and positive results, a cut-off value as three times the average values of negative control was calculated.

Statistical assessment: Statistical analysis of the data have been made by SPSS for Windows 10. In the tables, the values are means± standard deviations (sd). The differences between variants as compared to the control were analyzed with one-way ANOVA-LSD taking $P<0.05$ as significant.

3. RESULTS AND DISCUSSIONS

Observations on the in vitro cultures regarding the multiplications and the rooting process

Microshoots subjected to the action of alternating current were passed in optimum conditions the sterilizing stage and were grown *in vitro*, the initialization of multiplication process occuring in about 10 days. The culture aspect assessed visually was uniform, without vitrification or necrosis phenomena (Figure 1).

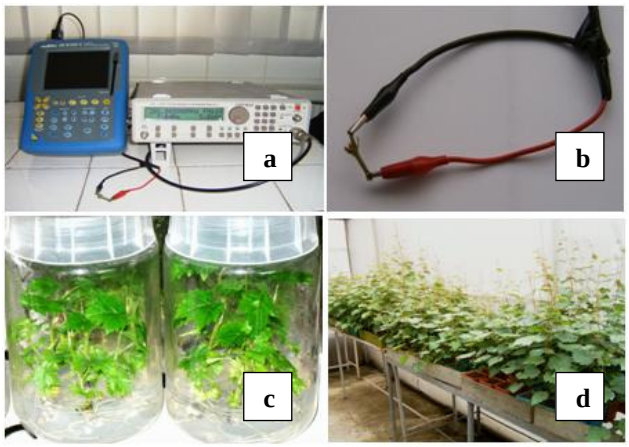

Figure 1. The electrotherapy with discontinous electric current experiment.

a) the device of electrotherapy; b) direct stimulation of the microshoot;
c) *in vitro* rooted grapevine plantlets; d) regenerated vines after electrotherapy

The evolution of the subcultures was assessed by the multiplication rate as compared to the control for each infected grapevine genotype, depending on the characteristics of alternating current. In the first subculture, the virus infected genotypes behaved no significant differences comparatively with untreated (controls); the multiplication rates range between 1.5000 ± 0.7071 and 3.0000 ± 1.4142. Starting from the second subculture, significant differences in the case of GLRaV-3–infected cv. Cabernet Sauvignon and ArMV+GFkV–infected unidentified genotype (1) have been occurred. În the first case, the difference was significantly negative at $P< 0.05$ for 10 000 kHz frequency as compared with the control. Also, negative significaton for the double infection ArMV+GFkV at 1000 kHz has beeen found.

After three subculture, the material has been stabilized; significant negative differeces appeared to genotypes Fetească albă, Cabernet Sauvignon, Frâncu ă and Grasă

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de Cotnari. The data regarding the multiplication rates on three subculctures of infected grapevine genotype indicated that the electrotherapy with alternating current of variable frequency influenced negatively the *in vitro* development of grapevine in some cases. This can have beneficial consequences for the purposes of possible viral inactivation multiplication due to intracellular thermotherapy phenomenon produced by electric current (Table 2).

Table 2. The evolution of *in vitro* culture on experimental variants expressed by the multiplication rate during three subcultures (I, II, III)

Grapevine genotype/ Virus	Frequency (kHz)	Multiplication rate (X)		
		I	II	III
Fetească albă / GFLV	1000	1.50 ± 0.7071	3.00 ± 0.0000	8.50 ± 4.9497 [b]
	10 000	1.50 ± 0.7071	5.00 ± 1.4142	17.50 ± 4.9497
	Control	1.50 ± 0.7071	5.50 ± 2.1213	23.00 ± 4.2426 [a]
Fetească neagră / GLRaV-1	1000	1.50 ± 0.7071	5.50 ± 3.5355	16.50 ± 10.6066
	10 000	3.00 ± 1.4142	6.00 ± 1.4142	20.50 ± 0.7071
	Control	2.50 ± 2.1213	3.50 ± 0.7071	15.50 ± 0.7071
Frâncușă / GLRaV-1	1000	2.00 ± 1.4142	1.50 ± 0.7071	3.15 ± 0.2121
	10 000	2.00 ± 0.0000	1.50 ± 0.7071	5.10 ± 0.8485 [b]
	Control	1.50 ± 0.7071	1.50 ± 0.7071	3.50 ± 0.7071 [a]
Cabernet Sauvignon / GLRaV-3	1000	1.50 ± 0.7071	3.50 ± 0.7171	2.90 ± 0.4243 [b]
	10 000	3.00 ± 0.0000 [b]	2.10 ± 0.2121 [b]	1.60 ± 0.2828
	Control	1.50 ± 0.7071[a]	4.70 ± 1.7678 [a]	1.45 ± 0.0070 [a]
Grasă de Cotnari /GFkV	1000	2.50 ± 0.7071	1.80 ± 0.2828	5.10 ± 0.1414 [b]
	10 000	3.00 ± 0.0000	2.30 ± 0.4243	4.60 ± 0.5303
	Control	3.00 ± 0.0000	1.80 ± 0.2828	3.60 ± 0.4950 [a]
Unidentified	1000	2.00 ± 0.0000	3.00 ± 0.0000 [b]	5.50 ± 0.7071

genotype (1) / ArMV +GFkV	10 000	2.00 ± 0.0000	4.00 ± 0.0000	7.00 ± 1.4142
	Control	1.00 ± 0.0000	6.00 ± 0.0000 [a]	6.50 ± 0.7071
Unidentified genotype (2) / GFLV+ GLRaV-1	1000	1.00 ± 0.0000	2.00 ± 1.4142	3.65 ± 0.4950
	10 000	1.50 ± 0.7071	2.50 ± 0.7071	3.25 ± 0.3536
	Control	1.00 ± 0.0000	2.00 ± 0.0000	3.50 ± 0.7071

The values are means± sd on three repetitions, the small letters represent the significance of the differences between variants as compared to the control at $P < 0.05$

Plant regeneration, in general, depends upon several factors, including: genotype, physiological state of the explant, culture medium, the cultivation conditions and the interactions betwen these factors (D. SVETLEVA & al. [18]). The electric pulses are olso reported as stimulant of ***in vitro*** plant differentiation (A. GOLDSWORTHY [19]). It was demonstrated that regeneration of potato plant tissues could be improved by exposing explants to mild electric current (H.F. LOZOYA-SALDAÑA & al. [10]).

Alternating electric currents (50 Hz, 0.1–50 μA) applied for 30 days on ***Nicotiana tabacum*** calli induced a significant stimulation of morphogenetic processes. The number of shoots increased by up to 300% for the samples stimulated with 50 μA but no significant changes were noted in total mass, DNA and protein content in the stimulated samples compared to control. It was suggested that in the presence of the external electric current, the callus cells become more sensitive to chemical signals (hormones and/or ions) in the culture medium (G. COGĂLNICEANU & al. [20]).

After three subculture on multiplication media, differentiated and elongated microshoots have been transfered on rooting medium. In general, the rhyzogenesis assessed by the number of rooted microshoots took place without large differences on the experimental variants. In the case of Fetească neagră cv. initially infected with GLRaV-1, a significant positive difference have been registered, in that the treatment with 10 000 kHz determined the increasing of the number of rooted shoots (121± 0.8112) comparatively with the control (69± 8.4853) (Table 3).

Significant increases in rooting rate and root growth parameter indicated that direct electric current applications may have the potential to improve propagation of grapevine rootstocks that are difficult to root by cuttings (C. Köse [21]). On the other hand, higher intensities of electric current adversely affected the survival of bean explants ***in vitro*** and, thus, the plant regeneration (M.H. HORMOZI-NEJAD [22].

Table 3. ***In vitro*** rooting assessed by the number of rooted shoots on the experimental variants

Genotype/ Virus	**Frequency (kHz)**	**Rooted shoots (N°)**
Fetească albă / GFLV	1000	11.00 ± 5.6569
	10 000	19.00 ± 4.2426
	Control	15.50 ± 0.7071
Fetească neagră / GLRaV-1	1000	71.50 ± 28.9914
	10 000	121.00 ± 0.8112 [c]
	Control	69.00 ± 8.4853 [a]
Frâncuşă /	1000	20.00 ± 21.2100

GLRaV-1	10 000	21.00 ± 19.8000
	Control	18.00 ± 2.8300
Cabernet Sauvignon / GLRaV-3	1000	13.00 ± 0.0000
	10 000	17.00 ± 1.4100
	Control	18.50 ± 2.1200
Grasă de Cotnari / GFkV	1000	36.00 ± 8.4900
	10 000	56.00 ± 29.7000
	Control	42.00 ± 2.8300
Unidentified genotype (1) / ArMV + GFkV	1000	6.00 ± 1.4142
	10 000	7.00 ± 1.4142
	Control	17.00 ± 1.4142
Unidentified genotype (2) / GFLV + GLRaV-1	1000	19.00 ± 1.4100
	10 000	20.00 ± 4.2400
	Control	19.50 ± 0.7100

The values are means± sd on three repetitions, the small letters represent the significance of the differences between variants as compared to the control at $P < 0.05$

Virus elimination assessment

The efficacy of virus elimination process assessed by ELISA testing of regenerated acclimated grapevine plants was presented in Table 4.

The analysis showed some encouraging results for elongated viruses GLRaV-1 and GLRaV-3 in single infections and no virus eradication in mixed infections had been obtained. The electrotherapy of GLRaV-1- infected material produced maximum 12.5% virus-free plants at 1000 kHz for and 4.5% GLRaV-3-free vines at 10 000 kHz, for 10 min of exposure. Grasă de Cotnari variety was 100% GFkV-free in the case of 10 000 kHz variant. The results regarding GLRaV-1 or GFkV elimination were not reproducible in the case of mixed infection containing one of them. In the case of 1000 kHz variants, any acclimated vine has been obtained for cv. Frâncu ă and unidentified genotype (2). No

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satisfactory results have been achieved for isometric viruses eradication (GFLV, GFkV) either in simple or mixed infections. ArMV has been eradicated in proportion of 85.7 – 88.9% but the vines belonging to unidentified genotype (1) remained GFkV infected.

Table 4. Assessment of efficiency of grapevine virus elimination by electrotherapy with direct electric curent

Grapevine cultivar / virus	**Frequency (kHz)**	**Plants (N°)**		**Sanitation rate (%)**
		ELISA tested (N°)	**Virus-free (N°)**	
Fetească albă / GFLV	1000	20	0	0
	10 000	17	0	0
Fetească neagră / GLRaV-1	1000	66	0	0
	10 000	146	0	0
	1000	8	1	12.5

	10 000	2	0	0
Cabernet Sauvignon / GLRaV-3	1000	8	0	0
	10 000	22	1	4.5
Grasă de Cotnari / GFkV	1000	15	0	0
	10 000	9	9	100
Unidentified genotype (1)/ ArMV+GFkV	1000	7	6- ArMV-free 0 -GFkV-free	0
	10 000	9	8– ArMv-free 0- GFkV-free	0
Unidentified genotype (2)/ GFLV+GLRaV-1	1000	-	-	-
	10 000	7	0	0

The succes of electrotherapy in producing virus- free plants depends upon both plant regeneration and virus elimination rates (M.H. HORMOZI-NEJAD [22].

Taking into consideration that both, antiviral treatment with electric current and *in vitro* culture, can be considered stress factors responsible for inducing genetic variation, it is compulsory to check the obtained plants for genetic stability, fidelity and uniformity. Regenerated grapevine after exposure to electrotherapy in continous electric field were morphologically identical to the control (non-treated plants), as shown in the studies comparing their random amplified polymorphic DNA (RAPD) profiles (I.C. GUȚĂ & al. [14]).

CONCLUSIONS

The influence of alternating electric current and the behaviour of grapevine genotypes in the presence of virus infection, during *in vitro* axillary buds proliferation, could not be correlated.

The behaviour of viruses in sanitation process is different in simple or mixed infections; electrotherapy in alternating current of single infected microshoots produced GLRaV-1-, GLRaV-3- or GFkV-free grapevine in different percentage, but no virus elimination in mixed infections containing one of these viruses has been obtained.

No satisfactory results have been achieved for GFLV eradication both in simple and mixed infections.

In order to increase the number of grapevine cultivars and clones needed to be available as healthy material, the certification program and, also, the virus-elimination method for obtaining virus-free grapevines must be constantly developed.

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EFFECT OF INORGANIC NITROGEN NUTRITION ON "IN VITRO" PRODUCTION OF POTATO MICROTUBERS

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Abstract: *The effect of inorganic nitrogen nutrition on production of microtubers was studied in two potato genotypes Nicoleta and Christian. The objective of this study was to investigate whether a reduction in total nitrogen level in the Murashige & Skoog medium would improve microtuberization. The effect of three levels of total nitrogen (30, 45 and 60 meq) on tuberization was studied at constant (20 meq K). Reducing the total nitrogen supply increased the number but decreased the size of microtubers. The weight of microtubers per vessel was the highest at the highest nitrogen concentration (60 meq): 1.91 g for Christian variety. A reduction in total nitrogen supply reduced the size of microtubers, the lowest weight being at the lowest nitrogen level – 30 meq: 1.01 g for Nicoleta variety, with a difference of -0.66 g, significant in a negative way. Decreasing the total nitrogen supply caused increasing the number of microtubers/ vessel which was the highest at the lowest nitrogen concentration (30 meq): 19.67 microtubers, with a difference of 4.34 microtubers, significant in a positive way, for Christian variety. The number of microtubers per vessel was the lowest at the highest nitrogen concentration (60 meq): 15.33 microtubers, for both varieties.*

Keywords: microplants, mineral nutrition, microtubers, *Solarium tuberosum* L., tissue culture

1. INTRODUCTION

Some cultivars are more sensitive than others to total nitrogen levels or relative concentration of nitrate: ammonium in the medium during micropropagation (Avila et al. 1998) or microtuberization (Garner and Blake 1989; Sarkar and Naik 1998). Low nitrogen in the micropropagation and microtuberization media was best for microtuberization (Stallknecht and Farnsworth 1979; Wattimena 1983). Total nitrogen and nitrate: ammonium levels were used effectively to improve preconditioning, induction (Charles et al. 1995; Zarrabeitia et al. 1997; Sarkar and Naik 1998), and microtuber growth (Chen and Liao 1993; Sarkar and Naik 1998). Reduced ammonium levels during source plant preconditioning resulted in increased numbers and better synchronization of subsequent microtuber induction (Charles et al. 1995; Zarrabeitia et al. 1997; Vreugdenhil et al. 1998).

The amount of nitrogen had an effect on microtuberization (Cutter, 1978; Moorby, 1978).

Stallknecht and Farnsworth (1979) and Wattimena (1983) found that low nitrogen in both the explants and the tuberization media was best for induced tuberization in potato. In the absence of growth regulating substances in the medium, a reduction in total nitrogen supply or an increase in the ratio of ammonium to nitrate reduced the size and number of microtubers (Garner & Blake, 1989).

Hoque *et al.*, (1996) reported that higher number of microtubers can be produced in MS medium by reducing $KN0_3$ by half of the total amount. Reduction in the amount of NH_4N0_3 to 1/8th of its amount present in MS medium, initiated early and higher microtuberization after 4 wk (Zarrabeitia et ai., 1997). Nitrogen is one of the major nutrient

elements that regulate plant growth and development. The form of nitrogen supplied to plants may influence the uptake of other nutrients (Schmitz and Lorz, 1990). A low level of ammonium supplied in combination with nitrate is favorable to tuber growth, but a higher level of ammonium supply is detrimental (Garner and Blake, 1989). In MS basal salt medium the two main sources of nitrogen (N): ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3).

The present experiment was undertaken to study the effect of inorganic nitrogen supply on the induction and development of potato microtubers "in vitro".

2. MATERIALS AND METHODS

Nicoleta and Christian potato *(Solanum tuberosum* L.) varieties were used in the experiment.

"In vitro" plantlets of potato were multiplied by subculturing of single stem nodes. The multiplication medium contained mineral salts and vitamins plus 0.05 mg/l NAA and 20g/l sucrose. The medium was solidified with 8 g/l Agar. Temperature in the growth chamber was 25 ^{o}C with 16 hr photoperiod and light by fluorescent tubes.

Fifteen explants (each with two nodes) were cultured for each treatment and cultivar, in vessels. The microtuberization medium is liquid and contains the same substances as medium Murashige-Shook (1962) coumarin, kinetin, sucrose (80-90 g/l). The stock solution of cumarine and kinetin were prepared as follows:

Cumarine: 500 mg in a 200 ml recipient; 25 ml ethanol are added; the solution is completed with distilled water.

Kinetin: 200 mg in a 50 ml recipient; 20 ml NaOH 1N are added; the solution is completed with distilled water.

For 1 l medium we add 20 ml stock solution of coumarine and 12.5 ml stock solution of kinetin. The microtuberisation medium doesn't contain agar. In every plastic recipient we poured 45 ml medium The microtuber induction cultures were incubated in the dark at 20^0C. We harvested the microtubers separately according to variety.

After the tuberization period (7-8 weeks of darkness), plantlets were extracted from cultures recipients, and harvested microtubers were washed, to avoid the subsequent infections, which can appear during their storage. The tuberization percentage (number of explants with at least one microtuber over the total number of explants cultured) was measured.

In nitrogen-rich MS medium the concentration of total inorganic nitrogen is 60 meq. In the present experiment, alterations in MS macronutrients were made to obtain three levels of total nitrogen (30, 45 and 60 meq) while keeping potassium level constant at 20 meq. The different media used in the experiment are shown in Table 1.

Observations were recorded on the number of microtubers per g vessel, average microtuber weight.

3. RESULTS AND DISCUSSION

Reducing the total nitrogen supply increased the number but decreased the size of microtubers. Reduced total availability of inorganic nitrogen in the medium caused a

decline in the average microtuber weight or size of the microtubers, suggesting that the mineral nitrogen nutrition is a major limiting factor controlling their size.

Table 1. Total of nitrogen used in nutrition medium

Nitrogen (meq)	Salts			
	KNO_3		NH_4NO_3	
	(mg l^{-1})	mM	(mg l^{-1})	mM
30	950	9.4	825	10.3
45	1425	14.1	1237	15.5
60	1900	18.8	1650	20.6

Higher numbers of microtubers could be obtained using MS medium supplemented with 30 meq total nitrogen, 20 meq potassium, 80 g 1^{-1} sucrose, but increase in microtuber numbers was associated with simultaneous decrease in microtuber size. For commercial seed potato production, microtuber size is more important than microtuber number because small sized microtubers are more vulnerable to storage loss (Naik & Sarkar, 1997) and are unsuitable for direct field planting (Jones, 1988).

Table 2. Influence of nitrogen concentration and on weight of microtubers produced/ microplant

Variety	Total nitrogen (meq)	Average weight of a microtuber (g)	%	Dif. (g)	Signif.
Nicoleta	60	1.68	100.00	-	
	45	1.25	74.91	-0.42	ns
	30	1.01	60.46	-0.66	o
Christian	60	1.91	100.00	-	
	45	1.49	78.50	-0.41	ns
	30	1.14	60.09	-0.77	o

LSD 5%= 0.60 g LSD 1%=0.80 g LSD 0.1%=1.03 g
ns= not significant

The weight of microtubers per vessel was the highest at the highest nitrogen concentration (60 meq) (table 2): 1.91 g for Christian variety and 1.68 g for Nicoleta variety. The lowest weight of microtubers was obtained at the lowest nitrogen level 30 meq: 1.14 g for Christian variety, with a difference of -0.77 g, significant in a negative way, and 1.01 g for Nicoleta variety, with a difference of -0.66 g, significant in a negative way.

The number of microtubers per vessel was the highest at the lowest nitrogen concentration (30 meq) (table 3): 19.67 microtubers, with a difference of 4.34 microtubers, significant in a positive way, for Christian variety, followed by Nicoleta with 18.33 microtubers and a difference of 3.00 microtubers, significant in a positive way.

The number of microtubers per vessel was the lowest at the highest nitrogen concentration (60 meq): 15.33 microtubers, for both varieties.

Table 3. Influence of nitrogen concentration and on number of microtubers produced/ microplant

Variety	Total nitrogen (meq)	Average number of microtubers/vessel	%	Dif. (no. of microtubers)	Signif.
Nicoleta	60	15.33	100.00	-	
	45	16.67	110.89	1.34	ns
	30	18.33	119.59	3.00	*
Christian	60	15.33	100.00	-	
	45	17.00	110.89	1.67	ns
	30	19.67	128.29	4.34	*

LSD 5%= 2.82 microtubers LSD 1%=3.88 microtubers LSD 0.1%=5.32 microtubers
ns= not significant

4. CONCLUSIONS

The experiment showed that mineral nitrogen nutrition strongly influenced the production of potato microtubers. The mineral nitrogen nutrition is a major limiting factor controlling their size.

Reducing inorganic nitrogen in the medium had effect in the average microtuber weight or size of the microtubers.

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IN VITRO *CONSERVATION* BY SLOW GROWTH OF *ARNICA MONTANA*

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Abstract. *The addition of osmoticums to the nutrient medium has proved efficient for reducing growth rates of different plant species. An efficient protocol for mass micropropagation of A. montana was developed, which is an essential requirement for prolonged in vitro storage. In vitro study of mannitol on conservation of A. montana plants was conducted. The plants were cultured on ½ Murashige and Skoog (MS) rooting medium containing 2% sucrose, 0.5 mg/l IBA and different addition of mannitol (0, 1, 2, 3 and 4%). The development of shoot and root growth as well as percentage of survival was evaluated during 1, 3 and 6 months of in vitro storage. It was found that ½ MS medium supplemented with 2% mannitol effectively retarded shoot and root length and number of formed roots. After six months the survival rate was 65%. A combination of mannitol and low light intensity was most effective in prolonging the term between subcultures. The survived plantlets regenerated new shoots after subcultivation onto the fresh propagation medium. An effective protocol for long-term in vitro conservation of A. montana in slow growth conditions was developed, allowing the storage of this endangered species in tissue culture.*

Keywords: ***Arnica montana, in vitro*** **conservation, mannitol, slow growth**

1. INTRODUCTION

In the last decades many valuable plant species are threatened with extinction due to unfavorable environmental conditions. Application of modern approaches to preservation of natural plant resources should be a major goal of researches. Conservation through traditional methods requires much efforts, area and funds (Charoensub and Phansiri, 2004). Plant material is vulnerable to natural disasters, climate changes, attacks by pests and diseases during cultivation. The conventional methods for propagation are slow and inclined to unprofitable losses. Therefore it is necessary to develop alternative methods for conservation of endangered and rare medicinal plants using plant tissue culture (Fay, 1994). The germplasm preservation allows reduction of the number of subcultures and maintains the genetic diversity of species in a sterile condition ensuring retention of plant stability (Moges et al. 2003; Shibli at al. 2000). The growth rate of *in vitro* cultures can be limited by various approaches including incubation at reduced temperature and low light intensity, changes in some media components, additions of osmoticums and growth retardants (Shibli et al, 2006). *In vitro* storage under slow-growth conditions were carried out for certain species like banana, potato, yam and cassava and were routinely used for protection of some valuable species (Ashmore, 1997).

Arnica montana L. is a well known medicinal plant spread in the mountains in various regions of Europe. It is a rare and endangered species widely applied in pharmacy,

homeopathy and cosmetics. The plant is used as anti-inflammation drug stimulating wound healing. It possesses also bacteriostatic, fungistatic, antirheumatic, cardiotonic and antihyperlipidemic effects (Willuhn, 1998). At present circumstances - law protected status and enhanced pharmacy interest it is necessary to elaborate methods for preservation of valuable clones of this species.

The objective of the present study was to develop an effective protocol for long-term *in vitro* conservation of *A. montana* under slow growth conditions allowing the storage of this endangered plant.

2. MATERIALS AND METHODS

Prolonged *in vitro* conservation of plant material: The *A. montana* shoots were obtained from *in vitro* seedlings of German population (Botanical garden, Chemnitz). The plant material was maintained on rooting medium (basal medium containing half-strength MS nutrients salts supplemented with 0.5 mg/l IBA) in order to align the phase of growth and development. Retarding the growth of cloned plants was accomplished through:

- Using the half-strength MS medium
- Reducing the concentration of sucrose
- Addition of osmoticum (mannitol)
- Cultivation under low light intensity (20 $\mu Mm^{-2}s^{-1}$)

For the experiment, *A. montana* plants were cultured on ½ MS medium containing 2% sucrose, 0.5 mg/l IBA and different addition of mannitol (0; 1; 2; 3 and 4%). The medium without mannitol was used as control. The plants were placed vertically in the glass tube (150 x 20 mm), containing 8 ml of rooting medium. One shoot per culture tubes was inoculated and each treatment involved 20 plants.The treatments were repeated two times. A survival of plants was monitored each month within six months without subcultivation. The survival of prolonged storage *in vitro* cultures was determined by the presence of green plants with healthy growing tips without necrosis. The percentages of rooted plants, height of vegetative part of plants and root length were recorded during storage (1, 3 and 6 month of culture).

Multiplication of *A. montana* plants after long-term storage plant material*:* The recovery of plants after prolonged cultivation on medium with mannitol was initiated by culturing under optimal conditions for growth and development. The plants were subcultured on two different propagation media (full strength MS medium supplemented with 1 mg/l BAP or combination of 1 mg/l BAP and 0.1 mg/l IAA). The mean number of shoots per explant after 3 and 6 months of *in vitro* conservation was recorded. The data were statistically analyzed using Sigma Stat computer package (Sigma Stat 3.1, Systat Software, San Jose, California, USA).

Culture conditions: All media were adjusted to pH 5.6 before autoclaving. The nutrient media were sterilized and autoclaved at 120 ºC for 20 min at 1 atm. The cultures were stored at 22 ± 2 °C (16/8h photoperiod) and low light intensity (20 $\mu Mm^{-2}s^{-1}$) for *in vitro* conservation of plant material and light intensity (40 $\mu Mm^{-2}s^{-1}$) for recovery of plants after prolonged cultivation.

3. RESULTS AND DISCUSSION

Prolonged *in vitro* conservation: The literature survey revealed that up to now the protocol for *in vitro* conservation of *A. montana* under slow growth conditions have not been developed. The main requirement for prolonged *in vitro* storage is an effective method for micropropagation, as described previously (Petrova et. al. 2005, 2011). Table 1 presents both optimal propagation medium and rooting medium for *in vitro* conservation of *A. montana* plants.

Table 1. Murashige and Skoog nutrient media modified for *A. montana in vitro* conservation

Nutrient medium composition	Nutrient media for *in vitro* conservation	
	MSP (for micro propagation)	MSR (for rooting)
MS	Full strength	Half strength
Sucrose (%)	3	2
Agar (%)	0.6	0.6
BAP (mg/l)	1.0	
IAA (mg/l)	0.1	
IBA (mg/l)		0.5
Mannitol (%)		2

The clonal propagation of plants can be saved valuable genotype, but the process needed plant subcultivation on fresh medium every months. *In vitro* conservation through retarding the growth allows subcultivation at significantly longer periods (several months to year), which is more economically profitable (Negash et al. 2001). In our study slow growth of plants was achieved by maintaining *in vitro* cultures in the presence of osmoticum (mannitol) at concentration 0; 1; 2; 3 and 4% and low light intensity (Table 2, Fig.1).

Table 2. Effect of different concentration of mannitol on survival rate of *A. montana* plants

Concentration of mannitol at ½ MS nutrient medium (%)	After 1 month		After 3 months		After 6 months	
	Survival, %	Rooting, %	Survival, %	Rooting, %	Survival, %	Rooting, %
0	100	100				
1	90	100	90	95		
2	65	80	65	70	65	55
3	55	40	40	35		
4	20	0				

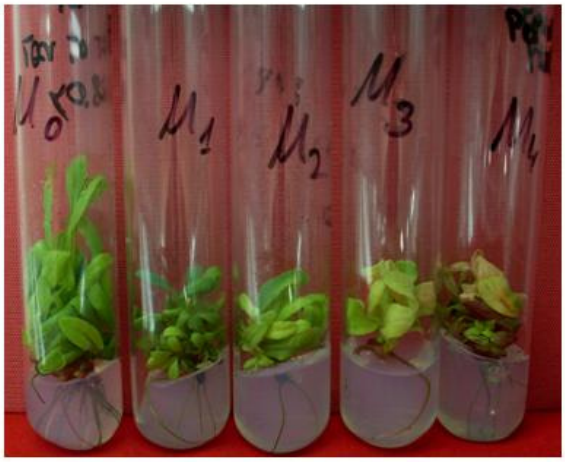

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Fig.1 *A. montana* plants maintened on ½ MS nutrient medium with different concentration of mannitol: a) Control; b) 1%; c) 2%; d) 3%; e) 4%

The plants cultivated on ½ MS rooting medium (control) formed well developed root system. The percentage of rooted plants reached 100% on the first month after inoculation. The plants needed a transfer to fresh medium because of their rapid growth (2.8 cm). When adding 1% mannitol to the medium the plants were viable and the percentage of survival and rooting was 90 and 100% respectively, after one month of cultivation (Table 2). The presence of 1% mannitol initiated formation of 2-3 adventives shoots at the base of plant. On the other hand this concentration of mannitol caused growth reduction of vegetative part of plants (average height 1.7 cm), but the length of roots remained almost the same as control (2.4 cm), allowing plants to be maintained for three months (Fig. 2). After this period of time subcultivation on fresh medium was required. The addition of 2% mannitol established condition of osmotic stress, which reduced the growth rate and produced short plants. Thus increased the time of subcultivation, but decreased values of all parameters investigated. This concentration of mannitol was retarded development of plants compared to control. The survival rate decreased to 65% in the first month and remained constant until the end of cultivation. The stems were shortened, the leaves were with small size and the development of root system was detained. This allowed subcultivations of plants after six months. The average height of plants was reduced to 1.1 cm. The differences between the heights of control plants and those cultivated on MS medium supplemented with 2% mannitol were well proven statistically ($P \geq 0.01$). The number of formed roots and their length were also reduced. The plants that didn't form roots required an earlier transfer to fresh medium. The combination of 2% sucrose and 2% mannitol in ½ MS medium provided slow plant growth and increased the storage period of six months. It was found that this combination is optimal for ***in vitro*** conservation of plants maintained under low light intensity. The further increasing of mannitol concentration led to yellowing of leaves

and necrosis of plant tips. The plants died after the third month. The content of 4% mannitol in the medium blocked the growth and development of plants. The survival rate was low (20%) and plants died in the first month after inoculation.

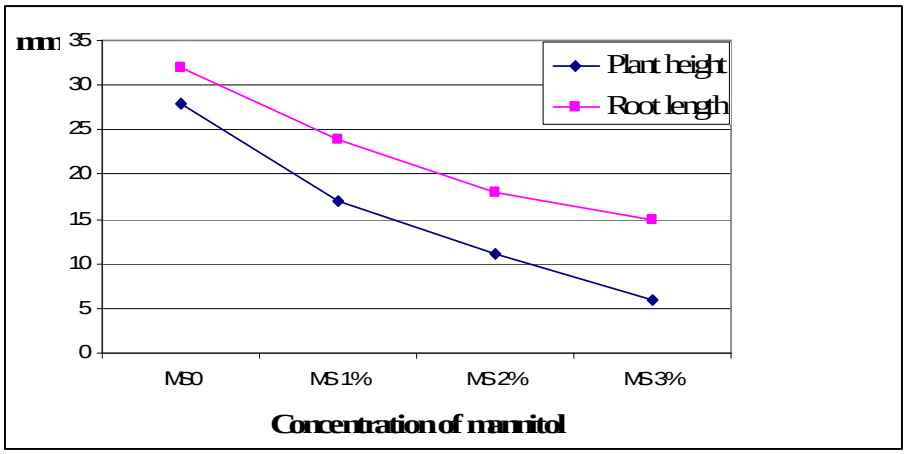

Fig. 2 Effect of mannitol on the plant height and root length of *A. montana* under conditions of slow growth

Multiplication of *A. montana* plants after prolonged *in vitro* conservation: *In vitro* cultures could be effectively maintained for 6 months. The plants were transferred to MS free medium without growth regulator and osmoticum in order to return towards normal growth and development. The plant growth was restored at a temperature of 22 ± 2 °C and light intensity of 40 $\mu Mm^{-2}s^{-1}$. The plants subsequently were micropropagated on MS media containing 1.0 mg/l BAP alone or in combination with 0.1 mg /l IAA after three and six months of storage. Shoots that had been incubated for 6 months multiplied slowly on multiplication medium under normal growth conditions. It was necessary one or two passages of plants subcultivation before started the process micropropagation. Basal part of plant tissue slowly expanded and induction of new vegatative buds was started. The increase of the swollen tissues and the formation of adventitious buds were more obviously in cultures grown on MS medium supplemented with 1 mg/l BAP and 0.1 mg/l IAA. On this medium also the higher micropropagation rate was observed in comparison with plants cultivated on MS medium supplemented with 1 mg/l BAP. The plants produced 2-3 new shoots (MS medium with 1 mg/l BAP) and 3.5- 4 shoots (MS medium with 1 mg/l BAP and 0.1 mg/l IAA) during sub-cultivation after three months of storage. The micropropagation frequency was significantly lower in plants maintained six months in terms of slower growth than in three months (Table 3). Since the height of the plants was reduced during storage and main axes of the shoots were in a passive state, when the plants were transferred to fresh medium for micropropagation they grew into viable buds. The plants were rooted successfully on ½ MS medium containing 0.5 mg/l IBA for rhizogenesis. Roots were observed within two weeks after the transfer of rooting medium.

Table 3. Micropropagation of *A. montana* after prolonged *in vitro* conservation with mannitol

Sub-cultivation	Number of shoots/explant After three months		Number of shoots/explant After six months	
	BAP (1 mg/l)	BAP (1 mg/l) + IAA (0.1mg/l)	BAP (1 mg/l)	BAP (1 mg/l) + IAA (0.1 mg/l)
1	0.8±0.12	1.2±0.18	0.6±0.09	0.8±0.13
2	2.7±0.22	3.5±0.27	1.8±0.14	2.2±0.18
3	3.4±0.25	4.0±0.26	1.9±0.15	2.3±0.20

The protocols of *in vitro* conservation were developed of few plant species. Mannitol at a concentrations of 20 g/l added to the medium reduced the growth of rose coloured leadwort plants and prolonged subcultivation eight months (Charoensub and Phansiri, 2004). Sarkar and Naik, 1998 reported that 20 or 40 g/l mannitol increased the survival of potato *in vitro* plants, but concentration of 60 g/l, caused death of plants. The effect of mannitol for *in vitro* storage of potatoes (Mix, 1985), strawberries (Vysotskaya, 1994), bananas (Van den Houwe et al. 1995) and enset (Negash et al., 2001) was reported.

4. CONCLUSION

Long-term storage of *in vitro* plant material of *A. montana* under slow growth conditions ensures the development of an effective system for protection and preservation of this endangered medicinal species. *In vitro* cultures could be effectively maintained on ½ MS medium supplemented with 0.5 mg/l IBA and 2% mannitol for 6 months at 20 $\mu Mm^{-2}s^{-1}$ light intensity. After prolonged storage the shoots were multiplied on MS medium supplemented with 1 mg/l BAP and 0.1 mg/l IAA. This protocol can be used for *in vitro* conservation of valuable *A. montana* clones.

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INFLUENCE OF PRE-SOWING TREATMENT OF SORGHUM SEEDS WITH MICROELEMENTS ON THE GERMINATION AND GROWTH OF PLANTS IN THE INITIAL STAGES OF THEIR DEVELOPMENT

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Abstract. *Container tests with sorghum seeds soaked for 24 hours in solutions with concentration 20mg/l of the following substances - $ZnSO_4$, $MnSO_4$, H_3BO_3, $CuSo_4$, $MgSO_4$, and control sample – with water - were carried out, after which 50 seeds were planted. The germination has been reported and after gathering of the test plants, the length of their roots and aboveground mass and the weight of the fresh and dried biomass.*

Based on the reported data it has been determined that the highest germination is present in the option treated with $MgSO_4$ and in the control sample (92%), followed by the options with H_3BO_3 (88%) u $CuSO_4$ (84%), and the lowest germination was with seeds, treated with $MgSO_4$ (80%) and $ZnSO_4$ (78%).

The pre-sowing treatment with $ZnSO_4$ and with $CuSO_4$ stimulates the development of the root system (respectively 49% and 20% longer than the control sample's roots), and with H_3BO_3 – of the aboveground mass (12% higher than the control samples).

Microelements stimulate the accumulation of more water in plant tissue.

Keywords: seeds, sorghum, microelements, germination

1. INTRODUCTION

Lately a lot of works from different scientists, showing the ability to increase yields of many plants by pre-sowing treatment of seeds with different microelements solutions, have appeared (Peeran, SN and Natanasabapathy, S., 1980; Khalid, BY and Malik, NSA, 1982; Sherrell, CG.,1984; Marcar, NE and Graham, RD (1986;). Some microelements have the ability to join up with specific proteins and form biocatalysts – vitamins and metallo enzymes, which stand out with their very high biological activity and play an important role in the growth and development of plants (Shkolnik, M. Y. and Makarova, N. A. 1957).

In the late 30s Russian scientists P.A. Vlasyuk and I.K. Onishtenko conduct the first research on the influence of Zn feeding on plants and prove its positive role. They determine that plants have the higher Zn needs in the early phases of growth and forming and development of the germ.

Bondarenko (1956) carried out tests with which he proves the great effectiveness of boron fertilization of carbonate black earth.

The issue of the influence of pre-sowing treatment of seeds with microelements on the activity of root micro flora also deserves attention. In this respect the experiments of Rahno (1950) which show an increase of microorganisms in the risosphere of roots, and with them of physiological processes of the plants, is of interest.

Some researchers (Kornilov, A.F. and Deeva V.P., 1955) think that pre-sowing treatment of seeds is a type of root fertilization but in fact pre-sowing treatment of plants

have the advantage before root feeding with the fact that each plant is provided with the needed quantity of any given microelement and those microelements are placed directly in the plant. This is an easy and cheap way of feeding up of plants in the initial stages of their development.

By treating seeds before sowing one can achieve not only an even feeding up of plants with microelements but also a reset of vital processes in the germ itself (Lorenz,1980; Agarwala S.C. and colleagues,1995; Hong F.S.and colleagues 1996;).

The purpose of this test is to research the influence of pre-sowing treatment of sorghum seeds with the following microelements – zinc, manganese, boron, magnesium, and copper on the germination and growth of plants in the initial stages of their development.

2. MATERIAL AND METHODS

This experiment was carried out in the Agricultural Institute – Shumen, where container tests were set with seeds for grain sorghum, Sadovo species, crop 2010. Seeds were soaked for 24 hours in water solutions with concentration 20mg/l of the following substances: $ZnSO_4$, $MnSO_4$, H_3BO_3, $CuSO_4$, $MgSO_4$ and control – with water, after which 50 seeds were planted at the same depth in vegetation containers with soil from the institute's test fields. Soil is carbonate black earth with high humus content and soil reaction from slightly alkaline to alkaline (pH – 7,7-8,1). Reserves of available phosphorus is weak (16,3 – 21,8 P_2O_5 на 100 gr.), and regarding content of potassium, soil is medium to well supplied (40,3 – 50,3 K_2O /100 gr.).

Plants have been grown to the 4-5 leaf phase. Germination was reported at the 7th, 8th, 9th and 13th day from seed sowing.

After gathering of test plants the length of their roots and aboveground mass as well as the weight of dry and fresh biomass were measured.

Statistical analysis was made by the border difference method (Zapryanov, 1983).

3. RESULTS AND DISCUSSION

First sprouts were registered on the 5th day after sowing of seeds treated with magnesium and the control sample. On the 6th day seeds treated with zinc and boron started sprouting, and on the 7th day – seeds treated with manganese and copper.

Figure 1 shows the dynamics of germination of seeds soaked in solutions of different microelements. Results registered on the 8th day after sowing show that pre-sowing enrichment of grain sorghum seeds with $CuSO_4$ in concentration 20mg/l has a suppressive effect on germination (16 of 50 sown seeds). The percentage of germinated seeds was highest in the control sample – from 50 sown seeds we have 43 germinated. With the remaining options the number of germinated seeds varies from 34 (treated with $ZnSO_4$) and 37 (treated with H_3BO_3). On the 9th day the germinated seeds in control container and in options fed up with manganese and boron are almost equal (respectively 44, 42, 43 germinated seeds) and the rest of the options keep their slower pace of germination.

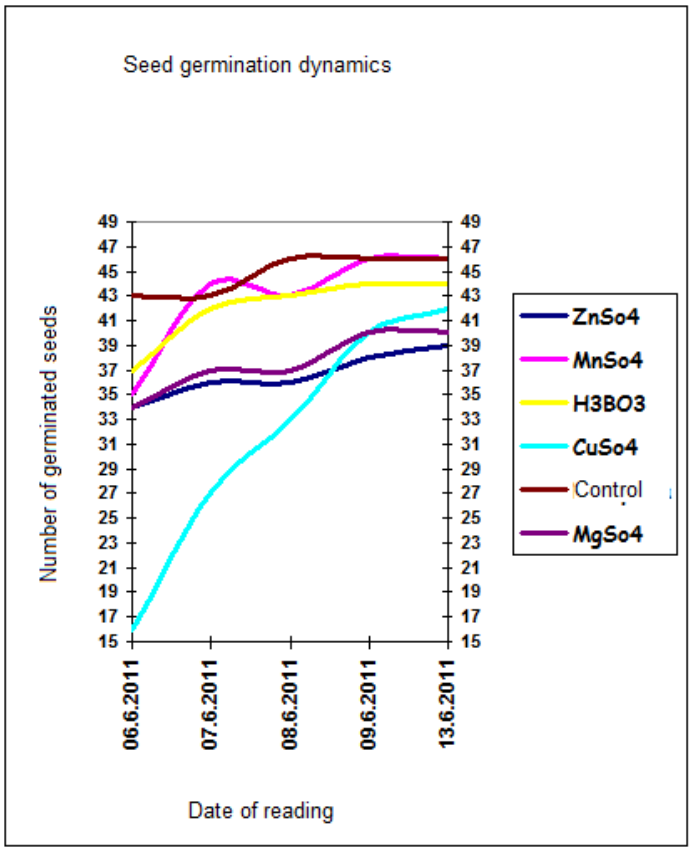

Fig. 1. The dynamics of germination of seeds soaked in solutions of different microelements

From 10th day until 14th day the dynamic of plumule growth increases in the option soaked in CuSO4 solution, and seeds from options wi th zinc and magnesium germinate slower.

During the last count of sprouts (13.06.2011) the highest germination was reported in seeds treated with $MnSO_4$ and in the control option, treated with water (92%) followed by H_3BO_3 option (88%) and $CuSO_4$ (84%).

The lowest level of germination was found in seeds treated with $MgSO_4$ (80%) and $ZnSO_4$ (78%).

According to Bulgarian Standards requirements the minimum germination level of pure sorghum seeds should not be lower than 80% and reported results (excluding samples treated with $ZnSo_4$) meet those requirements.

For the creation of a well-seasoned crop it is very important the simultaneous germination and sprouting of plants. Regarding this issue, microelements manganese and boron receive a positive mark.

Idres and Aslam (1975) and Shaban and Eid (1982) who have examined the pre-sowing treatment of wheat seeds with $ZnSO_4$, $CuSO_4$ and $MgSO_4$ and its effect on germination, report similar results.

Table 1 shows the results from statistic treatment of the reported data for average length of roots and above ground parts of different options.

Table 1 Average length of root system and above ground parts of plants from different options

Option	Average length of root cm	D	Sd	t	Proof	Relative %
$ZnSo_4$	30,09	9,83	2,27	4,33	***	149
$MnSO_4$	20,96	0,7	1,85	0,38	-	103
H_3BO_3	17,50	2,76	1,32	2,09	*	86
$CuSo_4$	24,21	3,95	2,02	1,96	-	120
$MgSO_4$	21,60	1,34	1,78	0,75	-	107
Control	20,26					
Option	Average length of above ground part cm	D	Sd	t	Proof	Relative %
$ZnSo_4$	19,74	0,13	0,98	0,13	-	101
$MnSO_4$	19,65	0,04	1,0	0,04	-	100,2
H_3BO_3	22,02	2,41	0,93	2,59	*	112
$CuSo_4$	20,98	1,37	1,06	1,29	-	107
$MgSO_4$	20,38	0,77	0,85	0,91	-	104
Control	19,61					
p 5%=2,02 p1%=2,70 p 0,1%=3,55						

Plants treated with $ZnSO_4$ and $CuSO_4$ have the best developed root systems.

The average length of their roots surpasses the root length of control plants respectively by 49% and 20% and the difference between the control sample and the $ZnSO_4$ option is statistically very well assured. Plants treated with H_3BO_3 have the least developed root system – 14% lower average length from that of control plants, and this difference is statistically proven.

The remaining two options ($MnSO_4$ and $MgSO_4$) do not register considerable differences with the control samples (3-7%).

Regarding the average length of above ground parts of plants, differences vary between 0,2% and 4%, best developed above ground mass compared to the control sample have plants from seeds soaked in H_3BO_3 (12% higher plants from those soaked in water). This difference is proven on statistical level.

Data from this analysis confirms results of Bozhenko (1956) who has studied the effect of pre-sowing treatment of seeds with $ZnSO_4$ in different crops and determines the stimulating influence of microelement Zn on plant's root system growth.

Table 2 shows the proportion of roots and above ground parts of plants from different options in percent. This proportion in control is closer to 100 (103% in favor of root system). Plants from seeds soaked in $MnSO_4$ and $MgSO_4$ solution have results similar to those of control plants (respectively 105% and 106% in favor of the root system. More substantial differences are reported in test plants treated with $MnSO_4$, H_3BO_3 and CuSO4. The average root length of ZnSo4 option exceeds the average length of above ground mass by 52%, and $CuSO_4$ – by 15%. With H_3BO_3 the values of average length of above ground mass are higher by 31%. These results show that Zn and Cu stimulate the development of root system and B stimulates the development of above ground mass.

Table 3 shows the results from the measured weight of roots and above ground mass in fresh and dried condition. Weight of fresh roots of all test plants is less (from 49% to 21%) from that of control plants, lowest values measured in plants treated with $ZnSO_4$ and $MnSO_4$. Fresh mass weight of options with $MnSO_4$, H_3BO_3, CuSO4 and MgSO4 exceeds that of control plants respectively by 6%, 50%, 23% and 28%. Only in treated with $ZnSO_4$ option the measured fresh weight of leafs and stalks is less by 11% from that of non-treated seeds.

Table 2 Proportion between root and above ground parts length in different options

Length, cm			
Option	Above ground mass	Root	Proportion %
$ZnSo_4$	19,74	30,09	152,00
$MnSO_4$	19,65	20,96	105,00
H_3BO_3	22,02	17,5	79,00
$CuSo_4$	20,98	24,21	115,00
$MgSO_4$	20,38	21,6	106,00
Control	19,61	20,26	103,00

Table 3. Weight of fresh and dry biomass

Option	Fresh mass				Dry mass			
	Average for 1 plant, g.				Average for 1 plant, g.			
	Root	Relative %	Above ground mass	Relative %	Root	Relative %	Above ground mass	Relative %
$ZnSo_4$	0,197	51	0,241	89	0,038	57	0,041	65
$MnSO_4$	0,219	57	0,286	106	0,045	67	0,045	71
H_3BO_3	0,290	76	0,409	150	0,059	88	0,059	94
$CuSo_4$	0,304	79	0,333	123	0,047	70	0,047	75
$MgSO_4$	0,292	76	0,375	138	0,042	63	0,052	83
Control	0,384	100	0,271	100	0,067	100	0,063	100

Weight of dry roots and above ground parts in all test plants treated with microelements is less from that of control plants.

It makes an impression that plants treated with $MnSO_4$, H_3BO_3, $CuSo_4$ and $MgSO_4$ have had a larger weight of leafs and stalks from the control sample in fresh condition, and after drying the weight of their above ground parts is less than control plants which means that microelements stimulate the accumulation of more water in plant tissue.

4. CONCLUSIONS

- Highest germination is registered in the option treated with $MnSO_4$ and in the control option (92%) followed by option with H_3BO_3 (88%) and $CuSo_4$ (84%).
- Lowest germination was registered in seeds treated with $MgSO_4$ (80%) and $ZnSo_4$ (78%)
- Pre-sowing treatment of seeds with ZnSo4 and with CuSo4 stimulates the development of the root system (respectively by 49% and 20% longer roots than control sample), and with H_3BO_3– that of above ground mass (12% higher plants than control samples)
- Plants treated with $MnSO_4$, H_3BO_3, $CuSo_4$ and $MgSO_4$ have a larger weight of leafs and stalks in fresh condition, and after drying the weight of their above ground parts is less than that of control plants, which means that microelements stimulate the accumulation of more water in plant tissues.

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SECTION III: FOOD BIOTECHNOLOGY

EVALUATION OF INULIN GELLING PROPERTIES IN NEW TYPES OF JELLIES

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Abstract. *Inulin-type fructans have become a topic of much interest for both the food industry and for researchers (Roberfroid, 2005). At high concentrations in water, above 25% for standard chicory inulin and above 15% for long – chain material, inulin has gelling properties (Glibowski, 2011). Therefore the objective of this research work was to evaluate effect of agar-agar and inulin on the structural properties of experimental gels.*
The research was carried out at the Latvia University of Agriculture, Faculty of Food Technology. In gel production the inulin powder was used to make mixed gels from the agar-agar and inulin. To prepare control samples the following recipe was used: agar-agar (2 g), sucrose (28 g), water (145 g), citric acid (1 g) and inulin powder (24 g). In the research amount of agar-agar was varied in the following range: 1.0; 1.5; 2.0; 2.5 g. Agar-agar water solutions were boiled for 5 minutes at the temperature of 100 ºC, then sucrose and citric acid were added to the solution and cooled down to 85 ºC. Then inulin powder was added, mixture stirred and kept for 5 minutes at the temperature 85 ºC. The solution was hot filled in polystyrene containers (150 ml), which were sealed with lids and cooled, down to 18 ºC.
The gel strength (hardness) was characterised by cutting force. Texture was determined using a Texture Analyser TA.XT Plus. Wire cutter (A/BC) was used to slice the jelly samples. Measurements were carried out on samples at room temperature on the next day after gel samples were prepared. Structure of agar-agar and inulin powder dissolved in water, control samples with agar-agar, and inulin, as well as gel samples with both polysaccaharides were analysed under the triocular microscope Axioskop 40. Pictures were taken by digital compact camera Canon PowerShot A620 via 16 ×10 or 16 ×40 magnification of the microscope
In experimental gel samples, inulin concentration influenced the strength of the gels. The decrease in hardness of the gels can be explained by different properties of inulin and agar-agar. The strength of the gels became weaker when inulin was used as agar-agar substitute.

Keywords: inulin powder, agar-agar, gels

1. INTRODUCTION

Jellies are not basic foods, but they are good complements to a diet if they are eaten in correct amounts (Figuerola et al., 2007). Jellies are high-energy products, meaning that the products are not suitable or advisable for people who have glycemic problems, obesity, diabetes, and cardiovascular diseases.

Sugar in jellies is essential to form not only to product sweet taste, but also the product structure and gives to product calories (Bayarri et al., 2004). Some polysaccarides can be used to replace sugar in different sweets obtaining products with lower calories.

Inulin is legally classified as food or a food ingredient, and not as additive in all countries where it is processed in a number of different ways for the health food market (Roberfroid, 2005, Frack, 2002; Kaur et al., 2002). Inulin has been defined as a polydisperse carbohydrate consisting mainly of $-(2\rightarrow1)$ fructosylfructose links (Roberfroid, 2005). The most common are: native inulin with an average degree of polymerization (DP) of 10–12, inulin containing short chain fractions (with DP from 2 to 10) and a high performance inulin with an average DP no less than 23 (Roberfroid, 2005).

Inulin offers technological properties for a wide scope of food applications as well as important nutritional benefits. At high concentrations (> 25% in water for native and > 15% for long-chain inulin) inulin has gelling properties and forms a participle gel networking after shearing (Kim et al. 2001, Glibowski and Wasko 2008, Glibowski, 2009; Glibowski and Bukowska, 2011). When inulin is thoroughly mixed with water, a white creamy structure is obtained that can easily be incorporated in foods to replace fat. One of is its fat mimetic property which is used in the production of yogurts with decreased caloricity and full fat taste (Guven et al., 2005) and in ice-cream (El-Nagar et al., 2002) production.

Inulin incorporation into baked goods allows the replacement of sugar, fibre enrichment and better moisture retention properties (Frack, 2002). Inulin has found an interesting application as low calorie bulk ingredient in chocolate to replace sugar. Inulin syrup also can be used to substitute sugar in low-sugar jelly formulations by obtaining different product quality (Kronberga et al., 2011; Kronberga and Karklina, 2011).

Agar has been used for many centuries as a high performance gelling agent (Armisen et al., 2000; Stanley, 2006). Agar forms one of the strongest gels. Its ability to produce clear, colourless, odourless, and natural gels without the support of other colloids has long been exploited by the food industry not only as a stabilizer and gelling agent but also in the manufacturing of confectionery (Barrangou et al., 2006).

Interactions between hydrocolloids may modify the gel characteristics. The polysaccharide differs from other hydrocolloids in property of its high gel strength. The synergistic effect of inulin with other gelling agents constitutes additional advantage in all these applications. In several products inulin, and especially long chain version, can even (partially) replace gelatine, starch, maltodextrin, and other stabilizers. As of present jelly structure properties affected by agar-agar and inulin have not been investigated decreasing the content of agar and increasing the content of inulin in jellies. The knowledge of the mixed gels as well as the stability of such gels becomes very important for the development new products.

Therefore the objective of this research work was to evaluate effect of agar-agar and inulin on the structural properties of experimental gels.

2. MATERIALS AND METHODS

Inulin granulated powder made from chicory root (Orafti GR produced by BENEO-Orafti, Belgium) was used in the experiments. Agar powder produced by AgarNordS, Estonia (E 406) was used for preparation of experimental jellies. Sugar (Dansukker, Denmark) and citric acid (E 330) were purchased in local retailing and added to experimental gels.

Gel samples were prepared using concentrations of inulin powder (23.5–25 g), agar-agar (1.0–2.5 g) sucrose (28 g) and water (145 g).

Agar-agar was swollen in cold water, and then the mixture was heated until agar-agar was dissolved in water. Agar-agar/water solution was boiled for 5 minutes at a temperature of 100 °C, and then sucrose and citric acid added to the solution. Solution was cooled down to 85 °C and inulin powder was added, mixture stirred and kept for 5 minutes at the temperature 85 °C.

Subsequently, the obtained agar–agar/water with inulin powder, sugar and citric acid was hot–filled in polystyrene containers (150 ml), which were covered with lids and cooled down to 18 °C. To compare results of experimental gels the control samples were prepared using only agar-agar or inulin powder. The analyses of the structure were carried out on the next day after all kinds of gel samples were prepared.

Gel strength (hardness)

The gel strength (hardness) was characterised by cutting force. The texture was determined by using the Texture Analyser Model TA.XT Plus (Stable Micro Systems, UK) equipped with a load cell of 50 kg. The gel samples were positioned on a platform and cut using wire cutter (A/BC). The testing conditions were cross-head movement at a constant speed of 1 mm s^{-1}, a trigger point of 0.09 N, distance 13 mm. The average values of ten measurements are reported.

Preparing of microscopic samples of inulin and agar-agar powder suspended in water, and experimental gels (Table 1)

Structure of agar-agar and inulin powder dissolved in water, control gel samples with agar-agar, inulin as well as gel samples with both polysaccaharides were analysed under the triocular microscope Axioskop 40 (epifluorescence, excitation 450–490 nm, emission >500 nm). Pictures were taken by digital compact camera Canon PowerShot A620 via 16×10 or 16×40 magnification of the microscope.

The samples of experimental gels were cut in a 5 µm layer by device Microm HM315. Preparations were placed on a glass slide.

Table 1. Recipes of the samples for analysis of microstructure and texture (g)

Ingredients	Samples					
	1	**2**	**3**	**4**	**5**	**6**
Agar	2	-	2.5	2	1.5	1
Inulin	-	24	23.5	24	24.5	25
Water	145	145	145	145	145	145
Sugar	-	-	28	28	28	28
Citric acid	-	-	1	1	1	1
Total	**147**	**169**	**200**	**200**	**200**	**200**

Microsoft Excel software was used for the research purpose to calculate mean values and standard deviations of the experimental data obtained in the research.

3. RESULTS AND DISCUSSION

For developing of new products that meet the people's needs for a non-sugar diet, most of the foods are prepared by substituting sugar with sugar replacers – other sweeteners which give lower calories (sugar gives 4 kcal while fibre – 2 kcal per gram of the product) (Commission Directive 90/496/EU, Commission Directive 2008/100/EU). Jelly is a product manufactured by cooking fruit juice with added sugar, glucose syrup and agar-agar (Figuerola, 2007).

Sugar serves as a preserving agent and gelling aid. For proper structure, jelly products require the correct combination of agar, sugar and glucose syrup (Tabata, 1999; Bayarri et al., 2004). In order to examine how the strength (hardness) of gel changes, the development of an optimum gel model is in progress without adding any extra taste, aroma or colour additives (e.g., fruit or berry juice).

Regarding the gel strength (hardness), the data of gel texture in the literature sources is insufficient. Even very minor changes in composition or processing variables can dramatically influence the textural properties of jellies (Kim et al., 2001, Matsuhashi, 1990, Panouille and Larreta-Garde, 2009). Table 2 shows the gel strength of experimental gel samples if the content of agar-agar is decreased and the content of inulin in jellies is increased. For example, the hardness of gel is 2.87±0.14 N (sample 1) if 2.5 g of agar–agar are added, but 0.31±0.02 N (sample 4) if 1.0 g of agar is added. The concentration of inulin powder was in the range from 23.5 to 25 g 100 g^{-1} in gel mix.

Table 2. Gel strength of experimental samples

Sample no	Amount of agar-agar, g 100 g^{-1}	Gel strength, N
1	2.5	2.87 ± 0.14
2	2.0	2.38 ± 0.12
3	1.5	0.96 ± 0.05
4	1.0	0.31 ± 0.02

In this case the gel strength is a strong function of agar-agar concentration in gels. The strength of experimental gels decreases with increase in the content of inulin in gels. In comparison with agar-agar gel, the mixed gels were more easily deformable and they had lower strength. The significance of these interactions could influence the functionality of agar-agar in jellies. The type of inulin powder used in experiment, and its concentration did not form stable gel. More concentrated samples with inulin powder (50 and 60 g 100 g^{-1}) have to be prepared for formation harder gels (Chiavaro et al., 2007).

Figure 1 and Figure 2 show the microscopic images of particle size of inulin and agar-agar powder suspended in water and experimental gels. Agar can form either transparent or opaque gels which are thermally reversible on heating and cooling (McClements, 2007). Inulin with a crystal structure undergoes dispersion and forms a suspension in a water environment at room temperature. Most of the crystals do not change their structure. The crystals which do not dissolve form a weak three-dimensional network which is strengthened by dissolved inulin (Glibowski and Pikus, 2011). Obtained results showed that after heating and cooling the agar–agar is swollen and it formed the gel structure The

crystals of inulin are not so swollen at 100 ºC temperature and retained similar structure after heating. The formation of partial gel was observed. At moderate temperature (60–70 ºC), the polymer chains of inulin may have active movement in the solution due to higher kinetic energy. This may help the inulin chain be better dispersed in solution. Thus the better contact between inulin chains becomes easier during cooling to form gel structure. At high temperature (100 ºC) the inulin chain may start to degrade into shorter chains by hydrolysis which makes it harder to form a gel (Kim et al., 2001).

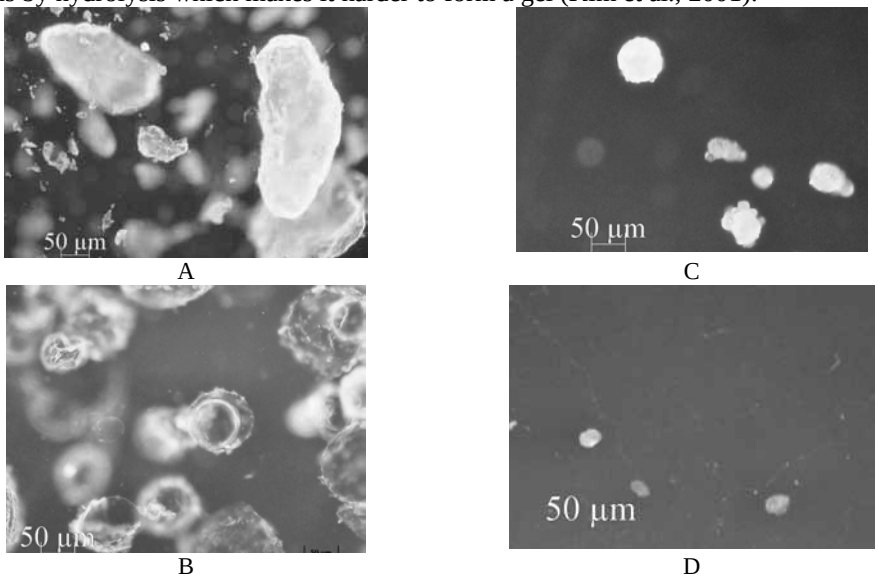

Fig. 1. Microscopic image of agar-agar and inulin (16 × 10 or 16 × 40):
A - powder of agar-agar; **B** – agar-agar heated in water at 100 °C; **C** - powder of inulin; **D** - inulin heated in water at 100 °C.

Similar results were obtained after analysis of the structure of gels of the experimental samples with microscope. In comparison with agar-agar gel, the mixed gels were much more deformable with some inulin crystals included in agar-agar gel (Fig. 2).

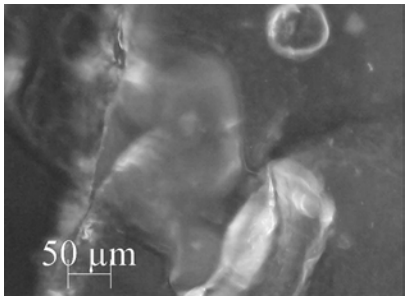

Fig. 2. Experimental gel sample with agar 2.5 g and inulin 23.5 g (16 × 10).

Decreasing the amount of agar-agar in experimental gels, much more inulin crystals could be observed in microscope and the gel became softer and it obtained smoother texture.
Gel formation is the key step to produce qualitative jellies. Our results show that the use of inulin decreasing amount of agar-agar in jellies the strength of the gels became weaker and formed insufficiently stable gel. The decrease in hardness of the gels can be explained by the different properties of inulin and agar-agar. Selected technological process for preparing jellies was not favourable for mechanism and factors of inulin gel formation.

4. CONCLUSIONS

From the data presented above we can conclude that in experimental mixed gel samples, with added inulin concentration influences the strength of the gels. Similar results are obtained analysing the microstructure of gels of the experimental samples. New technological process has to be developed to design new types of agar-agar/inulin enriched jellies with acceptable quality.

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GENETICALLY MODIFIED FOOD

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Abstract. *The variety of foods consumed by humans has changed greatly over the centuries, altering the balance of nutrients in the diet. Nutritional changes may have a more profound impact on the health of the population. Traditional plant breeding techniques of intra and inter-species crossing and mutation are designed to create genetic variation upon which selection of the most desired genotype is the expected outcome. All plant breeding procedures can produce unexpected effects. Generally consumers consider that traditional foods (that have often been eaten for thousands of years) are safe. When new foods are developed by natural methods, some of the existing characteristics of foods can be altered, either in a positive or a negative way. The purpose of this research was to establish the opinion of future food engineers (final year students) concerning the genetically modified organisms, so I asked them to complete a questionnaire of seven questions. The research conclusion was that more than 75% of them don't consider that the genetically modified organisms use is safe for consumption. However most of them don't really know what genetically modified organisms are so their opinion is not endorsed but is revealing for the lack of public information in this field.*

Keywords: genetically modified, food, safety.

1. INTRODUCTION

For more than ten years, genetically modified crops are cultivated worldwide for their increased yield, smaller production costs, higher quality, etc. In our country there are legally cultivated only 5 varieties of genetically modified corn according to the Romanian Official Varieties of Crop Plants Register, 2011 edition, while the genetically modified soybean production was stopped in 2007. Worldwide genetically modified soybean is the most cultivated crop, probably because of the market demand. Soybean products are found in a wide range on the Romanian market, whether is raw or as an ingredient. Romanian annual loss is almost 1 billion euro because it's not cultivating genetically modified soybean, the national production potential being about two million tons, has declared in a press conference, the Minister of Agriculture Valeriu Tabără."Romanian annual import of soybean from Brazil, U.S.A. and another countries is about 500 000 tons, and it is all produced only through biotechnology. We are currently attempting to get the approval for genetically modified soybean cultivating starting 2012 because the economic loss is significant. Among all genetically modified crops cultivated worldwide, only four rule the market: soybean, cotton, corn and rape. Regarding the cultivated surface, soybean is more spread. In 2009 more than three quarters (77%) from the 90 million hectares cultivated with soybean worldwide were genetically modified, while for cotton, almost half (49%) from the 33 million hectares were genetically modified. More than one quarter (26%) from the158 million hectares cultivated worldwide with corn were genetically modified and 21% of the 31 million hectares of rape cultivated worldwide were genetically modified (James, 2009). This study aims to identify the products than contain genetically modified soybean.

2. MATERIALS AND METHODS

The material was gathered using extensive literature and Internet searches and the research method was made through a questionnaire supported by approximately 100 persons that answered at a series of seven questions about the genetically modified organisms, from a consumer point of view. The persons that answered the questions were mostly final year students in food industry field, studying in Bucharest.

3. RESULTS AND DISCUSSIONS

Across the world, food is a part of cultural identity and societal life, and has religious significance to people. Therefore, any technological modification, including changes to the genetic basis of crops or animals used for food, may be met with social resistance. In many countries, people's interaction with nature, often correlated with religious perspectives, causes social and ethical resistance to modifications that interfere with genes. Whereas the objectives of food safety in its limited sense are more clearly realized and harmonized internationally, the objectives of nature protection, environmental safety and sustainable agriculture are much more complex, unclear and variable in different regions of the world.

The potential direct health effects of GM foods are generally comparable to the known risks associated with conventional foods, and include, for example, the potential for allergenicity and toxicity of components present, and the nutritional quality and microbiological safety of the food. It has been argued that random insertion of genes in GMOs may cause genetic and phenotypic instabilities (Ho 2002) but, as yet, no clear scientific evidence for such effects is available. A better understanding of the impact of natural transposable elements on the eukaryotic genome may shed some light on the random insertion of sequences. Food allergies or hypersensitivities are adverse reactions to foods triggered by the immune system. Within the different types of reactions involved, non-immunological intolerances to food and reactions involving components of the immune system need to be differentiated. The former may invoke reactions such as bloating or other unpleasant reactions, but are thought not to involve the immune system and called 'food intolerances'. The application of modern biotechnology to crops has the potential to make food less safe if the newly added protein proves to cause an allergic reaction once in the food supply.

Investigations of public perception in areas of the world with relatively high resistance to GM foods indicate that lack of information is not the primary reason (Lewenstein 2002; Birner and Alcaraz 2004). The public is not for or against GMOs *per se* — people discuss arguments both for and against GMOs, and are aware of contradictions within these arguments. Also, people do not demand zero risk. They are quite aware that their lives are full of risks that need to be balanced against each other and against the potential benefits. People may also discriminate in their perception of different technologies where a general positive perception can be observed for applications with a clear benefit for society, e.g. for modern medicines. A key finding is that people do not react so much to

genetic modification as a specific technology, but rather to the context in which GMOs are developed and the purported benefits they are to produce.

Knowing the genetically modified organism controversy I wanted to find out the fresh food engineers (final year students) opinion regarding this issue, so I developed a questionnaire of seven questions and I asked them to complete it. The questionnaire contained the next questions: what do you know about the genetically modified organisms? Are you aware of any food containing genetically modified organisms? Would you eat genetically modified food? What genetically modifies crop is now cultivated in our country? What do you think about the animals that are being feed on genetically modified fodder? Would you buy genetically modified food? Do you think that genetically modified organisms are safe to use?

To this questionnaire answered more than 100 food engineering students. At the first question meaning: "what do you know about the genetically modified organisms?" the percentage of the answers were:

- 20% don't know what the genetically modified organisms are;
- 17% think that they are harmful for our health;
- 33% have a wrong opinion about it;
- 30% had a clue about it and its purpose.

The second question "Are you aware of any food containing genetically modified organisms?" proved to be a real challenge, because the ones that answered correctly mentioned soybean, potatoes and corn although there aren't on the market and the others think that there are currently on the market genetically modified: chicken, sausage, fruits and vegetables. The percentage was this way:

- 20% doesn't know any genetically modified food;
- 31% gave a wrong answer indicating fruits or vegetables;
- 49% answered soybean or corn.

To the third question "Would you eat genetically modified food?" the percentage was:

- 47% gave a positive answer;
- 43% gave a negative answer;
- 10% couldn't decide.

In our country since 2007 the only genetically modified crop that is cultivated is corn MON810. To the question" What genetically modifies crop is now cultivated in our country?":

- 31% gived a wrong answer saying that genetically modified soybean, tomatoes, potatoes, canola and bees are now cultivated in our country;
- 40% answered correctly;
- 17% answered partially correct;
- 12% don't know anything about it.

Another delicate subject is about the animals that are being feed on genetically modified fodder, compound of genetically modified soybean and corn. So when I asked the student what they think about it 49% think that it can affect our health or the animal; 30% think is beneficial and 21% don't know a thing about it.

In my last study of the market I found that there isn't any genetically modified food on the Romanian market and knowing that I asked them also if they would buy genetically modified food, 3% said that they can't decide; 35% answered "yes"; 45%

answered "no"; 13% said that they have probably bought it without knowing and 7 % said that they would buy it if it doesn't have any side effects.

Although the safety of genetically modified organisms usage is still a controversy subject because there are conclusive studies on each part, at the question: "Do you think that genetically modified organisms are safe to use?" 76% answered "no"; 14% answered "yes" and 10% "don't know".

4. CONCLUSIONS

GM foods currently available on the international market have undergone risk assessments and are not likely to present risks for human health any more than their conventional counterparts. However the consumers opinion seems to be different, most of them consider that genetically modified food can affect their health.

Although there are lots of studies and World Health Organization reports about the genetically modified organisms the information seems to be hard to get because as we discover in the questionnaire most of the consumers think that the fruits, the vegetables and meat products are genetically modified and think that they are already eating this kind of food without being informed.

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**** HG 173/2006 privind trasabilitatea si etichetarea organismelor modificate genetic si trasabilitatea alimentelor si hranei pentru animale, obtinute din organisme modificate genetic;*

****http://www.madr.ro/pages/raport/agricultura-romaniei-noiembrie-2010.pdf*

****http://www.madr.ro/pages/page.php?self=01&sub=0104&art=0405&var=010402 consultat in data de 01.07.2011*

PHYSICAL-CHEMICAL PROPERTIES OF MONASCUS METABOLITES

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Abstract. *Monascal metabolites are widely using as food colorant in Asia, Europe and United States. Bioprocesses, based on solid state or submerged biosynthesis [1-3] are recommended as natural food dyes, but the safety product is insufficiently solved, due to content of citrinin. Citrinin is a mycotoxin especially toxic to liver and kidney tissue and is suspected of being a renal carcinogen leading to renal tumors. Used two strains we obtained the biopigments which are characterized by UV-VIS, and HPLC, in order to establish the major compounds in* Monascus metabolite and the mycotoxin concentration in them. The main conclusion of our study is the following: metabolites obtaining in submerged biosynthesis were a content of mycotoxin less than 3 ppm and is suitable to use as raw material in food industry

Keywords: biopigment, food safety, mycotoxin content

1. INTRODUCTION

Monascus extracts composition was not standardized; we know only that these extracts contain monacolins, meviolins, vitamins, polyphenols, azaphylones, gamma aminobutiric acid and ascorbic acid. Certain study performed on metabolites from *Monascus ruber* and *Monascus purpureus* reveal a content of 25 ppm meviolin, 17 units OD azaphylones (pigments), 15.6% proteins, 7.1% lipids, 1.3% monacolin K and 8% humidity [1-2]. According to study published in 1996 in Technique and protein Chemistry VII [3], metabolites obtained on solid substrates with *Monascus rubidigosus*, contain glucoamylase and carbohydrate in the following quantities: 43.9 mol mannose/ mol glucoamilase, 12.2 mol N-acetylglucosamina /mol glucoamilase and 8 mol galactose/mol glucoamilase [1].

Regarding the monacolins content, this is a function of strain type. From this point of view, studies performed on 7 types of *Monascus* strains revealed a content of Monacolin K between 128-7633 mg/kg [2]. But if we analyse the correlation between Monacolins and citrinin content, we see that only two strains were qualified for commercial use, respectively those in which the citrinin content is zero (figs.1-2). The content of azaphylone in *Monascus* metabolites were quantified by UV-VIS measurements, at 370 or 400 nm (yellow pigments), 420 nm (orange pigments) and 500 nm (red pigments) (fig.3). Studies performed by Espinoza [2] on metabolites obtaining in submerged media indicate a content of 2.5 g/L red pigment and 1.43 g/L orange pigment.

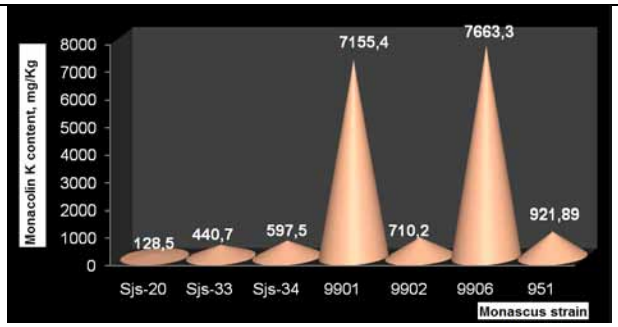

Fig. 1. Content of Monacolin K in bioproducts derived from 7 *Monascus sp*. Strains

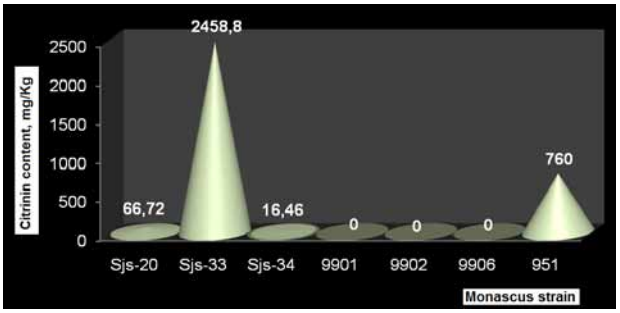

Fig. 2. Content of citrinin in bioproducts derived from 7 *Monascus sp*. Strains

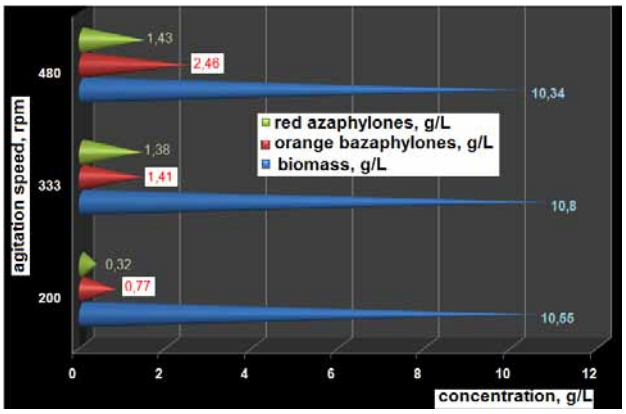

Fig. 3. Content of azaphilones in bioproducts obtained from *Monascus sp*. In submerged culture media which conyain 5% wheat flour, whitout gluten,at pH =5.6

According to studies performed by Yuan, Lin and Wang [2] content of gamma aminobutyric acid (GABBA) is of ten times more great in bioproducts obtained on solid

culture media in comparison with bioproducts obtained with the same strain , but in submerged culture (figure 4). The aim of our study is to quantify the content of azaphylone in Monascus metabolite obtained in submerged media and the citrinin, in order to indicate the suitability to use Monascus metabolites as food additive

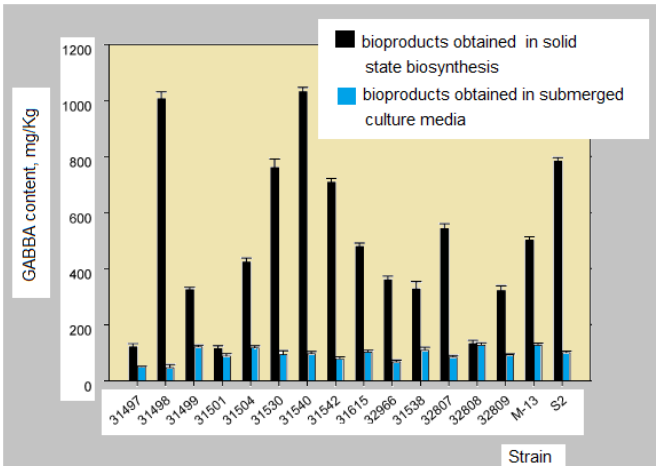

Fig.4. Content of gamma aminobutyric acid in bioproducts obtained with *Monascus sp*. on solide state or submerged biosynthesis

2. MATERIAL AND METHODS

In this study we used 2 strains of *Monascus*: *Monascus sp.1* and *Monascus sp.2* supplied by Bioengineering and Biotechnology Department of University POLIEHNICA of Bucharest. Metabolites from the two strains were obtained in submerged biosynthesis, with three culture media which contain starch (Medium 1), ethanol (medium 2) and dextrose (medium 3). The content of each culture media used in this study is presented in Table 1. The azaphylone content was monitorised during all bioprocess (10 days) by measuring the optical density of aqueous extract at 400 nm (for yellow azapylones), 420 nm (for orange azaphylones) and 500 m respectively (for red azaphylones). All experiments was realised in triplicate, on the thermostated Heydolph rotary shaker, in Erlenmeyer flasks with total volume of 500 ml. Each Erlenmeyer flask was filled with 100 ml culture media. In addition for all samples resulted from biosynthesis on dextrose media, the citrinin content was measured by higy performance liquid chromaotography (HPLC). The device used was the Agilent type, with C18 column. The solvent used as carrier was the methanol. Calibration curve was recorded with methanolic citrinin solution (the citrinin was supplied by Sigma).

3. RESULTS AND DISCUSSION

In culture media which contain starch, *Monascus sp.1* do not produced pigmentation. For the second strain *Monascus sp.2*, maximum concentration of red

azapylones was obtained after 106 h, (figure 5) with a OD=3.8 units units, whilst maximum concentration of orange compounds was obtained after 105 h, when a OD = 4.2 was obtained (figure 6.)

Table 1. Culture media used o obtain *Monascus* metabolite in submerged media

Culture media with starch		Culture media with ethanol	
Starch, g/L	30	Glutamic acid, g/L	5
Peptone, g/L	4	K_2HPO_4, g/L	5
glutamic acid, g/L	1	KH_2PO_4, g/L	5
bidistiled water	up to1L	$MgSO_4 \cdot 7H_2O$, g/L	0.5
Culture media with dextrose		$CaCl_2$, g/L	0.5
Dextrose, g/L	30	$FeSO_4 \cdot 7H_2O$), g/L	0.5
$NaNO_3$, g/L	0.1	$ZnSO_4 \cdot 7H_2O$, g/L	0.01
$MgSO_4$, g/L	0.05	$MnSO_4 \cdot H_2O$, g/L	0.03
$FeSO_4$, g/L	0.01	Ethanol, g/L	20
$ZnSO_4$, g/L	0.08	bidistiled water	up to1L
$MnSO_4$, g/L	0.03		
bidistiled water	up to1L		

Maximum concentration for yellow azaphylones was attain in 114 h with a OD = 2.85 (figure 8.)

In media with etanol the behaviour of *Monascus sp 1* is the same like in the case of starch media (without pigment production). In the case of strain *Monascus sp.2* , azaphylone appear, but in the concentration less than that obtained in starch culture media. So, in this case the productions of azaphylones red and yelow attain the maximum concentration after 145h, corresponding to an OD=1(figure 8 and figure 10), whereas the content of orange azaphylone attain a maximum concentration after 135 h with an OD = 0.27 (figure 9.

In submerged media which contain dextrose, the both strains produce azaphylones, but content of their is less than azaphylone concentration obtained in starch or etanol culture media. The Monascus sp. 1 strain give a maximum concentration of red azaphilones after 160 h, when obtain an OD =0.75 (figure 11). After 164 h, a maximum concentration of orange pigment is obtained (figure 12) with an OD =1. Two hour later (at 166 h) a maximum concentration of yellow pigment was obtained; with an OD =1 (figure 13),

Monascus sp. 2 give a maximum concentration of red azaphylones after 153 h, with an OD =0.75 (figure 14). After 166 h of biosynthesis, a maximum concentration of orange pigment was obtained (figure 15) with an OD=1. After that, at 177h a maximum concentration of yellow pigment was obtained, with an OD =1.2 (figure 16)

Fungal mycotoxin formation in submerged media. Fungi from Monascus genus produce one mycotoxin: citrinin. Content of this toxic compound in food, raw materials for food industry or fodder depend of Monascus strains and culture media.

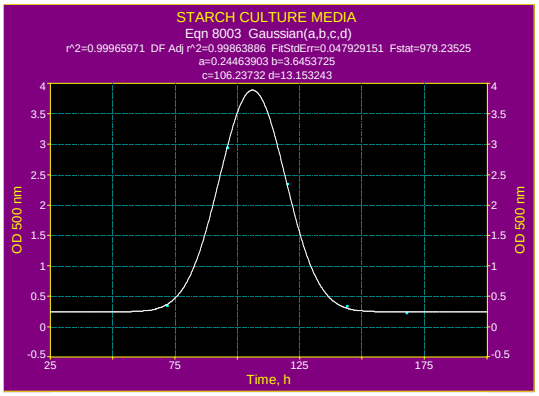

Figure 5.
Red azaphylone production during the biosynthesis in submerged media with starch and *Monascus sp.2.* Measurement performed at 500 nm

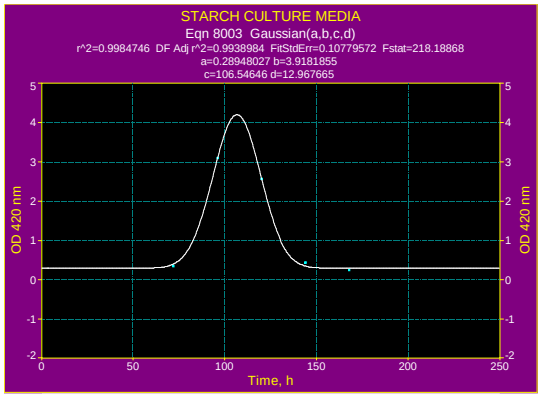

Figure 6.
Orange azaphylone production during the biosynthesis in submerged media with starch and *Monascus sp.2.* Measurement performed at 420 nm

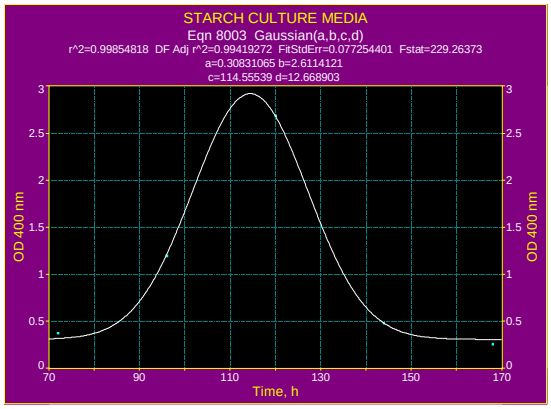

Figure 7.
Yellow azaphylone production during the biosynthesis in submerged media with starch and *Monascus sp.2.* Measurement performed at 400 nm

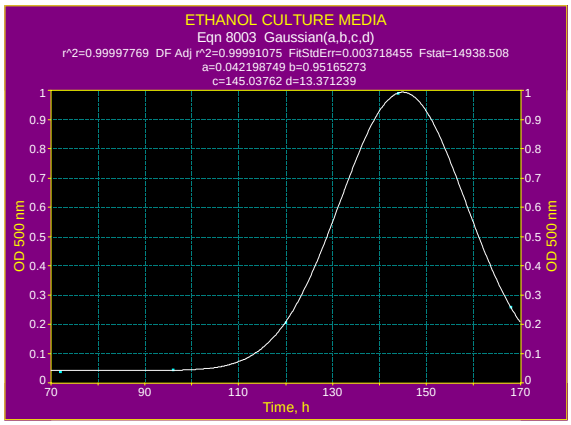

Figure 8
Red azaphylone production during the biosynthesis in submerged media with ethanol and *Monascus sp.2.* Measurement performed at 500 nm

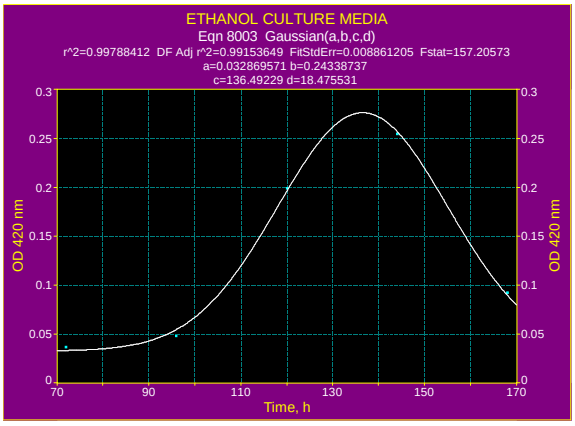

Figure 9
Orange azaphylone production during the biosynthesis in submerged media with ethanol and *Monascus sp.2.* Measurement performed at 420 nm

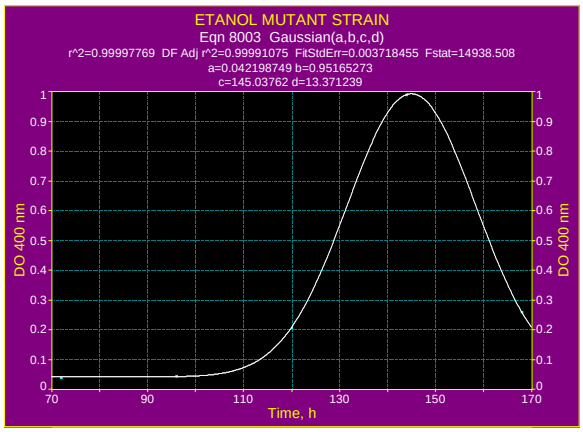

Figure 10
Yellow azaphylone production during the biosynthesis in submerged media with ethanol and *Monascus sp.2.* Measurement performed at
400 nm

If *Monascus purpureus* was developed on grain milk, the content of citrinin in culture media which result after fermentation is 386 mg/L [4-5]. If the same strain was growth on potato-glucose medium, the citrinin content at the end of fermentation is less than 1.1 ppm.

If we reffering to raw materials used in food industry, maximum concentration of citrinin find in cereals was 224.6 ng/g.

In some products used in animal fodder, sometimes citrinin concentration is bigger that 405 ppb [5].

In the case of *Monascus sp.1* which develop in culture media with dextrose, the study regarding the content of citrinin in liquid culture media indicate a maximum concentration at 96 h of biosynthesis, when the content of citrinin from the system attain 7.72 ppm. After 120 h of biosynthesis, the content of citrinin from the system decreased to 3.5 ppm (figure 17) and continue to decrease until 2,68 ppm at 240 h of biosynthesis.

This fact is positive because the maximum concentration of red azaphilones was attained after 200h, when in the system the citrinin concentration is less than 2.7 ppm. In this case results obtained are comparable with citrinin content from juice or wine.

In the case of strain Monascus sp. 2, the analysis indicate a maximum concentration of citrinin of 24.48 ppm, at 120h of biosynthesis. After 168h the content of citrinin from the system decreased at 3.39 ppm figure 18). After 10 day of fermentation, the content of citrinin from the system decreased to 2.2 ppm.

If we compare the time at which we obtain the maximumum content of azaphylone with time in which we obtain the minimum content of mycotoxin, we obtain an optimal time of biosynthesis situated in the range of 200-220h.

4. CONCLUSION

Good results are obtained in the case of biosynthesis of azaphilone on dextrose culture media using Monascus sp.1 or Monascus sp.2 as strains. In the last case, the maximum concentration of red azaphilones are obtained after 170 h of biosynthesis, (OD =1 for red azaphilones) when the content of citrinin is situated under 3 ppm. These results recommend the strains *Monascus sp.1* and *Monascus sp.2* to obtain food additives (pigments) in submerged culture media with dextrose.

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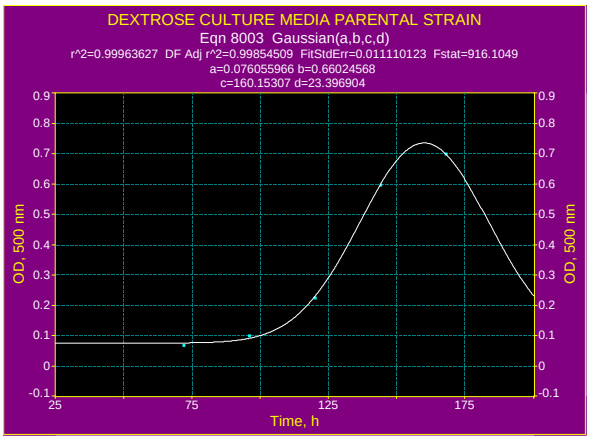

Figure 11. Red azaphylone production during the biosynthesis in submerged media with dextrose and *Monascus sp.1*. Measurement performed at 500 nm

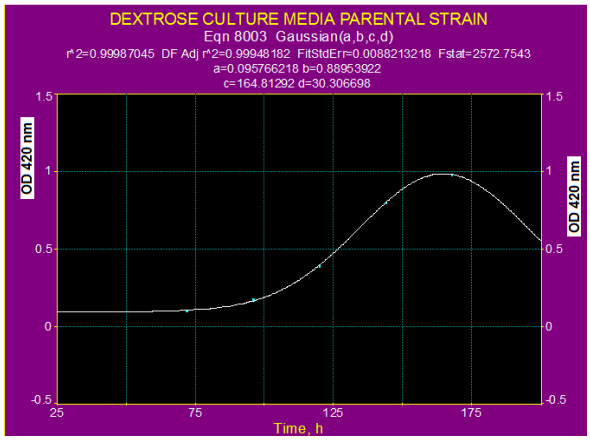

Figure 12. Orange azaphylone production during the biosynthesis in submerged media with dextrose and *Monascus sp.1*. Measurement performed at 420 nm

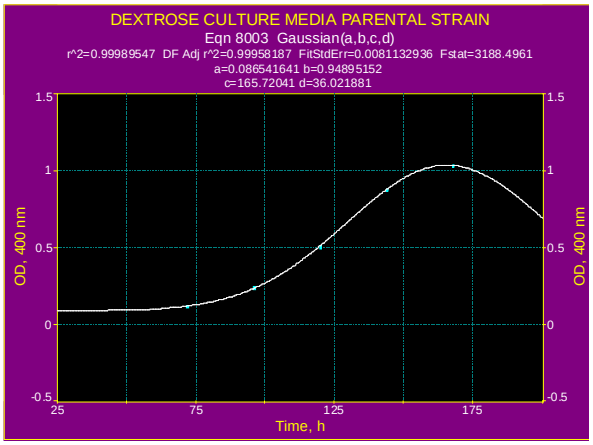

Figure 13. Yellow azaphylone production during the biosynthesis in submerged media with dextrose and *Monascus sp.1*. Measurement performed at 400 nm

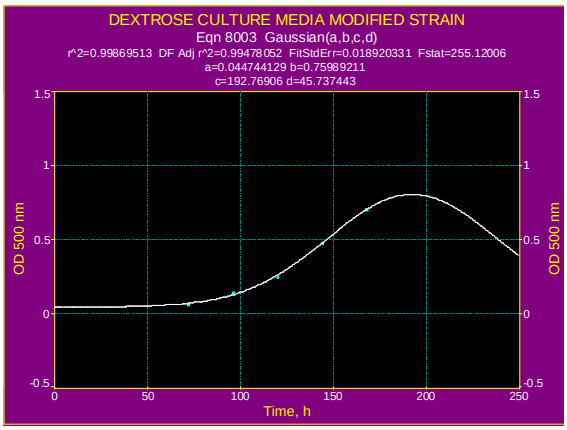

Figure 14. Red azaphylone production during the biosynthesis in submerged media with dextrose and *Monascus sp.2.* Measurement performed at 500 nm

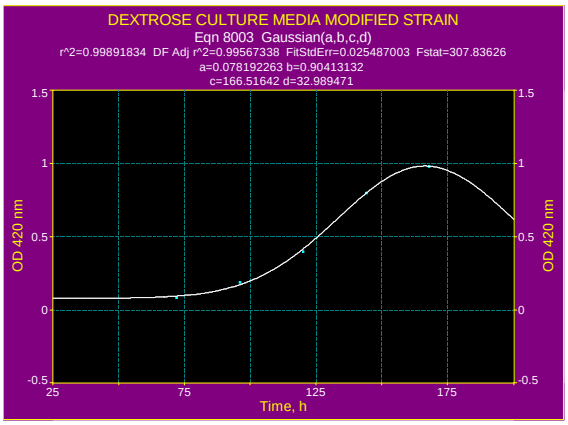

Figure 15. Orange azaphylone production during the biosynthesis in submerged media with dextrose and *Monascus sp.2.* Measurement performed at 420 nm

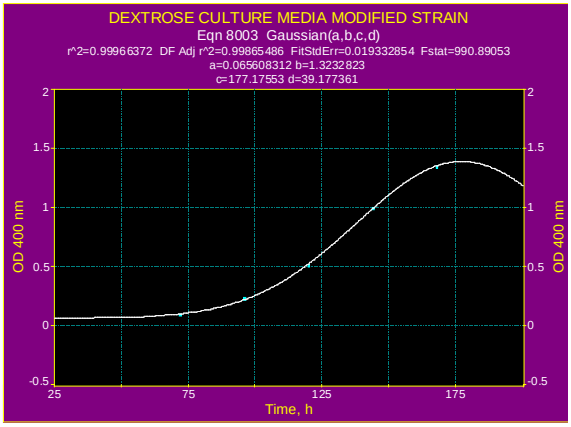

Figure 16. Yellow azaphylone production during the biosynthesis in submerged media with dextrose and *Monascus sp.2.* Measurement performed at 400 nm

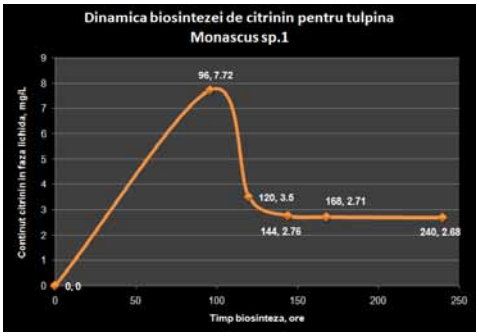

Figura 17 Citrinin production during the biosynthesis in submerged media with dextrose and *Monascus sp.1*

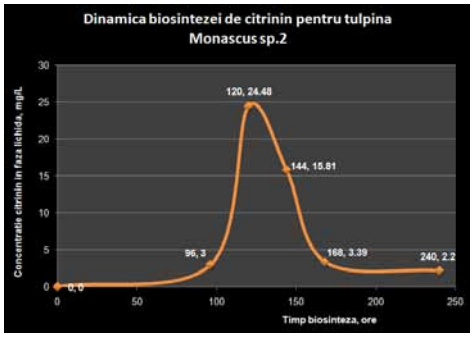

Figura 18 Citrinin production during the biosynthesis in submerged media with dextrose and *Monascus sp.2*

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THE EFFECT OF PREPARATES BASED ON SODIUM CARBONATE AND CITRIC ACID ON THE PARAMETER FALLING NUMBER IN HYPERDIASTAZIC FLOURS

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Abstract. *The objective was to assess the influence of compounds of sodium carbonate and citric acid on the amylase activity of hyperdiastazic flour. Four samples of wheat, with Falling Number values between 88 sec and 153 sec, were ground on pilot mill. Flour was treated with varying amounts of sodium carbonate/citric acid preparates and combinations. Results showed that the sodium carbonate preparate provides a significant improvement of Falling Number values at doses above 50 g per 100 kg, and citric acid increases Faling Number values of flour at doses greater than 200 g/100 kg. Combinations of the two, generally cancel this effect. Consequently, the use of sodium carbonate to improve Falling Number parameter values is limited because it is necessary to maintain acid pH at which bakery processes occur.*

Keywords: wheat flour, sodium carbonate, citric acid, falling number

1. INTRODUCTION

Environmental factors such as climatic conditions of the year, the area of culture, doses of nitrogen applied and fungicide treatments, influence the values of the Falling Number parameter of wheat, protein quantity and composition. The Falling Number parameter, known as Hagberg Falling index, reflects the amylasic activity of wheat and flour. More events were associated with low Falling Number, namely: late maturating wheat, which leads to reduced starch content, a lower quality of gluten and excessive rainfalls that cause the starch detachment of proteins [1].

Fungicide treatments and Fusarium infections have been incriminated in certain studies for an easy decrease of the value of this parameter [2]. For some varieties of wheat, the low values of the Falling Number parameter were correlated with a number of genetic disorders, characterized by continuation of α-amylase synthesis in the late maturation stages of wheat grains. For most of these varieties, the phenomenon seems to be modulated by the thermal shocks (freezing), to which the wheat plant is exposed in late stages of development [3, 4].

Romanian wheat yields are generally characterized by relatively small values of amylasic activity, often having higher Falling parameter values, higher than 350 seconds [5]. However, periodically, there are situations when the quality of crops is influenced by adverse weather conditions, especially during harvest. This leads to premature germination of seeds and the increase of amylasic activity. A big amylasic activity of wheat is a problem, as it leads to obtaining improper flour technological properties. Improving solutions are generally based on the change of dough pH, but have a limited effect, due to the sensitivity of biochemical and rheological processes in dough to pH changes. Another

line of research, which aimed to use amylasic inhibitors, has a limited applicability, due to their interference with the human digestive amylase.

2. MATERIAL AND METHODS

Four wheat samples taken from the 2010 harvest were analyzed in order to determine the amylasic activity through the Falling Index (ISO 3093:1997).

For each of the four samples we analyzed the Falling Number on the full meal (milled on the LM 120 laboratory mill taken from Perten Instruments AB) and the flour type 550 (obtained by grinding the samples on a Chopin CD1 pilot mill).

The obtained flour samples were treated with successive quantities of Rowelit (preparation based on 80% sodium carbonate, produced by Mühlenchemie), citric acid and combinations from them, in accordance with the experimental plan in Table 1.

Table 1. The quantities of Rowelit and citric acid used for treatments

Sample number	ROWELIT (g/100 kg)			CITRIC ACID (g/100 kg)			ROWELIT + CITRIC ACID (g/100 kg)				
I. II. III. IV.	50	100	200	50	100	200	25 + 25	50 + 50	100 + 100	25 + 100	100 + 25

The results have been interpreted using the methods of descriptive statistical analysis and t test (Student) for pairs of samples.

3. RESULTS AND DISCUSSION

The results obtained by Falling Number parameter determination, of the wheat samples and the flour produced from them, are presented in Table 2.

Table 2. Falling Number values and the estimates of their variability for the wheat and flour samples

Sample number	Wheat (s)	Wheat flour (s)
I.	132	158
II.	153	171
III.	113	125
IV.	88	120
Mean	**121.5**	**143.5**
St. dev.	27.671	24.906
C.V. (%)	17.356	18.791

The studied wheat samples had low values of the Falling Number parameter, well below the permissible limits of acceptability for their exploitation in specific technological processes of the milling and bakery industry (at least 180 s). However, we can see that the

milling process improves significantly this parameter, the average values for flour being with with 22 seconds higher than the average values for wheat (+18.1%, t = 5.003, p = 0.015).

Table 3 shows the values of the Falling Number parameter and the estimates of their variability in the flour samples treated with the preparate based on Rowelit sodium carbonate.

Table 3. Falling Number values and their variability estimates for the flour samples treated with citric acid Rowelit

Sample number	Rowelit			Citric acid		
	50g/ 100kg	100g/ 100 kg	200g/ 100kg	50g/ 100kg	100g/ 100 kg	200g/ 100kg
I.	177	181	219	163	158	179
II.	190	211	220	166	166	173
III.	136	149	183	118	115	141
IV.	130	148	182	119	121	139
Mean	158.250	172.250	201	141.5	140	158
St. dev.	29.736	30.037	21.370	26.589	25.729	20.944
C.V. (%)	18.791	17.438	10.632	18.791	18.378	13.256

The addition of Rowelit determines very significant increases of the values of Falling Number parameters, from untreated flour. The increase of the parameter value is almost linear (Figure 1), being 10% higher compared with the control when is treated with 50 g/100 kg (t = 5.990, p = 0.009), 20% higher compared with the control when is treated with 100 g / 100 kg (t = 7.367, p = 0.005) and 40% higher compared with the control when is treated with 200 g/100 kg (t = 19.438, p = 0.003). The enzymatic inhibition mechanism is probably based on the change of dough pH from the slightly acidic domain, that corresponds to the optimum pH of amylasic activity, to the neutral or slightly alkaline pH.

The addition of citric acid in doses of up to 100 g / 100 kg flour does not induce significant changes in the value of amylasic activity. In these treatments there have been noticed a slight increase in amylasic activity, showed by the decrease of the Falling Number parameter, compared to the control (-1.4% and -2.5%). The optimum pH of the amylasic activity in wheat is generally in the range 5.2 - 5.6, and a slight decrease, caused by the addition of citric acid may explain these results. However, treatments with bigger quantities of citric acid (200 g / 100 kg flour) decrease significantly the alpha-amylasic activity of flour (+ 10.1% compared to the control, t = 3.379, p = 0.040).

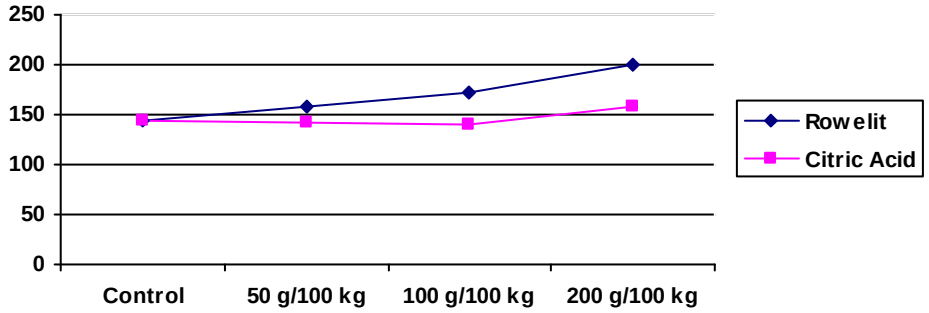

Figure 1. The evolution of the Falling Number parameter in case of treatments with Rowelit and citric acid

Since in bakeries using a wide range of acidifiers (acetic acid, lactic acid, citric acid etc), the use of sodium carbonate can be a problem, due to its reactivity. Table 4 contains the results obtained by the treatment of the flour samples with different combinations of the two preparates, namely Rowelit and citric acid.

Table 4. Falling Number values and their variability estimates for the flour samples treated with Rowelit and citric acid

Sample number	Rowelit + Citric acid				
	25 + 25 g/100kg	50 + 50 g/100 kg	100 + 100 g/100kg	25 +100 g/100kg	100 + 25 g/100kg
I.	158	165	166	175	195
II.	173	185	189	175	189
III.	123	125	121	120	139
IV.	116	120	118	121	138
Mean	**142.500**	**148.750**	**148.500**	**147.750**	**165.250**
St. dev.	**27.404**	**31.456**	**34.799**	**31.468**	**30.988**
C.V. (%)	**19.231**	**21.148**	**23.434**	**21.298**	**18.752**

From Table 4 we notice that the use of equal quantities of the two preparates did not alter significantly the value of the Falling Number parameter. Also, the combination of 25 g/100 kg Rowelit and 100 g/100 kg citric acid does not significantly influence the amylasic activity of flours. Instead, the combination of 100 g/100 kg Rowelit and 25 g/100 kg citric acid causes a significant increase of the Falling Number parameter (+ 15.16% compared to the control, $t = 4.207$, $p = 0.024$). This fact suggests that the use of average doses of sodium carbonate can be considered for reducing the amylasic activity in flours with defects, while the technological processes use relatively small quantities of acidifiers. However, the degree of inhibition of amylasic activity is not big enough to allow the correction of some flours having initially large amylasic activity. In the context of this research, we consider that the potential of reducing the amylasic activity by using sodium

carbonate is limited, for the flour or wheat samples having initially Falling Number greater than 140 seconds.

4. CONCLUSIONS

Our results show that the addition of sodium carbonate significantly decreases the amylasic activity of flours, which is valued by the Falling Number parameter. A similar effect can be achieved by using big doses of citric acid. Using combinations of equal quantities of citric acid - sodium carbonate does not significantly influence the amylasic activity of flours; this suggests that in the technological processes using acidifiers, the use of sodium carbonate has a limited applicability. However, the average doses of sodium carbonate combined with low doses of citric acid can be used for a slight decrease of amylasic activity. The effectiveness of treatment with sodium carbonate depends on the initial value of amylasic activity of flour; that is why we consider that this method can be used only for wheat flours having the value of the Falling Number parameter bigger than 140 seconds.

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INFLUENCE OF THE CULTURE MEDIUM ON TORULARHODIN PIGMENT BIOSYNTHESIS

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Abstract. *The aim of the present work is to study the formation of the intracellular carotenoid pigment - torularhodin depending on culture medium composition and other cultivation factors. Culture media have a considerable influence on the yeast biomass accumulation and carotenoid pigments biosynthesis, particularly torularhodin, component with a high-level antioxidant potential. The paper presents the influence of cultivation conditions on the growth and torularhodin formation of the yeast Rhodotorula rubra ICCF 209.*

Keywords: carotenoids, torularhodin, *Rhodotorula rubra*, β-carotene

1. INTRODUCTION

Amongst pigments of natural origin, carotenoids seem to play a fundamental role, their presence in the human diet being considered positively because of their action as pro-vitamin [1-3], antioxidant or possible tumors-inhibiting agents [4].

The major carotenoid pigments obtained by biotechnological methods are torularhodin, β-carotene and torulene produced in various concentrations by *Rhodotorula* and astaxanthin produced by *Phaffia rhodozyma* [5-7].

Culture media have a considerable influence on the yeast biomass accumulation and carotenoid pigments biosynthesis, particularly torularhodin component with a high-level antioxidant potential.

The aim of the present work is to study the formation of the intracellular carotenoid pigment - torularhodin with the yeast *Rhodotorula rubra* ICCF 209 depending on culture medium composition and different cultivation factors.

2. MATERIAL AND METHODS

The experiments were carried out in 1000 mL conical flasks, with 200 mL culture medium, on a rotary shaker (Gerhardt Laboshake) at 250 rpm, during 5-6 days of discontinuous aerobic bioprocess, at 28 ^{o}C in most cases, for the carotenoids mixture formation, mainly containing the torularhodin, in the stationary phase of the yeast *Rhodotorula rubra* ICCF 209 growth curve. The medium composition, defined as MS3, was obtained by previous research work [8] with the formula: 40 g/L glucose, 1.5 g/L yeast extract, 5 g/L NH_4NO_3, 1 g/L KH_2PO_4, 0.4 g/L $MgSO_4$ x7H_2O and 0.4 g/L NaCl. Trace elements are assumed to be taken from the tap water.

A suspension of the yeast cells in sterile water was used for the inoculum preparation. Inoculum was analyzed in terms of number of cells / mL.

The cells growth was quantified by: Optical Density (O.D.) determination at Λ = 600 nm, evolution of pH and dried biomass concentration. Dry matter concentration determination was done after biomass separation from the culture medium by centrifugation. The biomass drying was achieved in the oven at 105 ^{o}C until constant mass.

After cells' separation by centrifugation three freeze-thaw cycles were performed. The pigments extraction procedure was done in accordance with the dedicated literature [9], comprising acetone extraction of the total pigments mixture including water soluble species, followed by n-hexane extraction to separate the total carotenoids content; another extraction with alkaline methanol allowing the torularhodin (the only pigment with acid structure) component isolation.

The total carotenoids concentration and the torularhodin concentration were determined based on the spectrometric recording of the extracts on the spectrophotometer UV-VIS (Jenway Spectrophotometer).

To calculate the torularhodin concentration, the specific absorption coefficient $E^{1\%}$ 1932 was applied to the difference between the absorbance of the hexane extract before and after methanol phase extraction, at 515 nm [10].

3. RESULTS AND DISCUSSION

1. Influence of the initial pH

Several experiments were realized to determine the influence of the initial cultivation pH, in the range 3 – 8, on the yeast growth and both – total carotenoids and torularhodin formation, considering the control medium MS3 with initial pH 5.

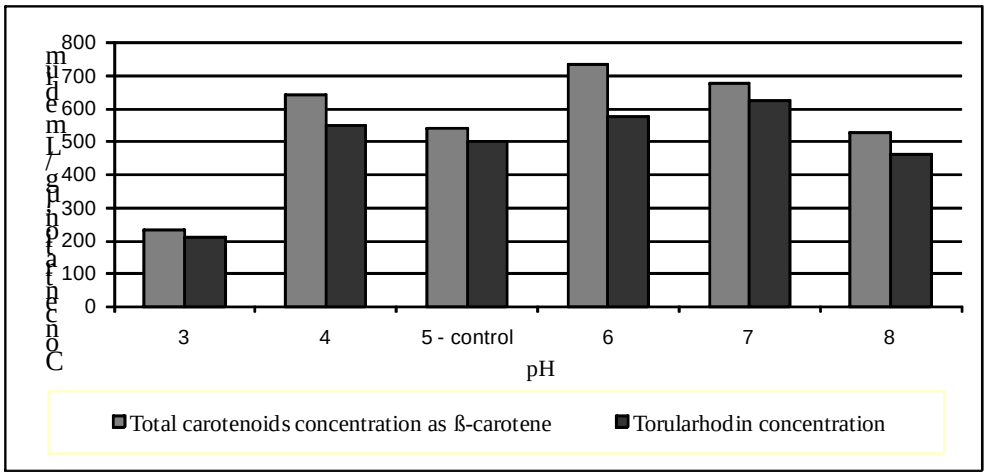

Fig. 1 Variation of total carotenoid and torularhodin concentration depending of initial pH values

The studied characteristics – yeast growth, pH evolution, the total carotenoids formation and the torularhodin formation recommend the initial pH range of 6-7 as being favorable, the torularhodin fraction from the total carotenoids' content being more than 90% for the

pH of 7. The pH 3 is not favorable for the pigments formation and the pH 8 is limiting the yeast growth.

2. Influence of the initial inoculum's concentration

Also the growth and the carotenoids production, mainly torularhodin with the same yeast ***Rhodotorula rubra*** ICCF 209 were investigated for different initial inoculum' cellular density on the control medium MS3. The growth evolution is done in the fig. 3 and the pigments formation results are presented in the fig. 2.

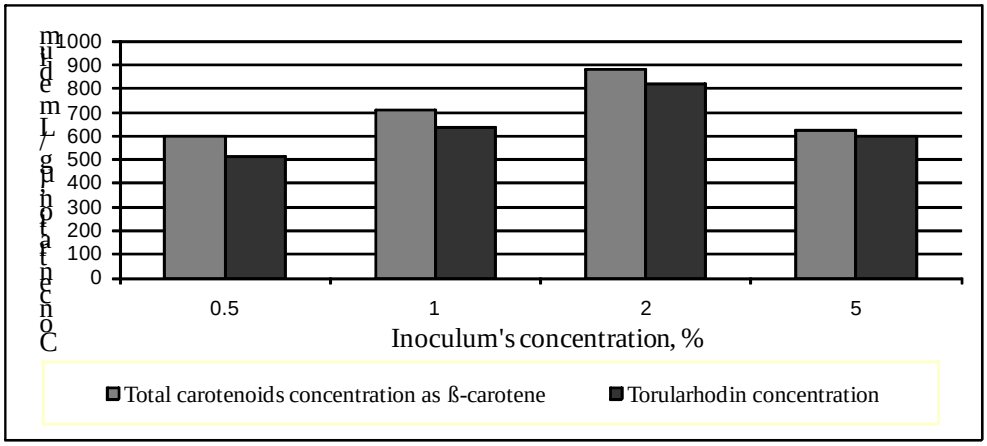

Fig. 2 Variation of total carotenoid and torularhodin concentration depending of the inoculum size

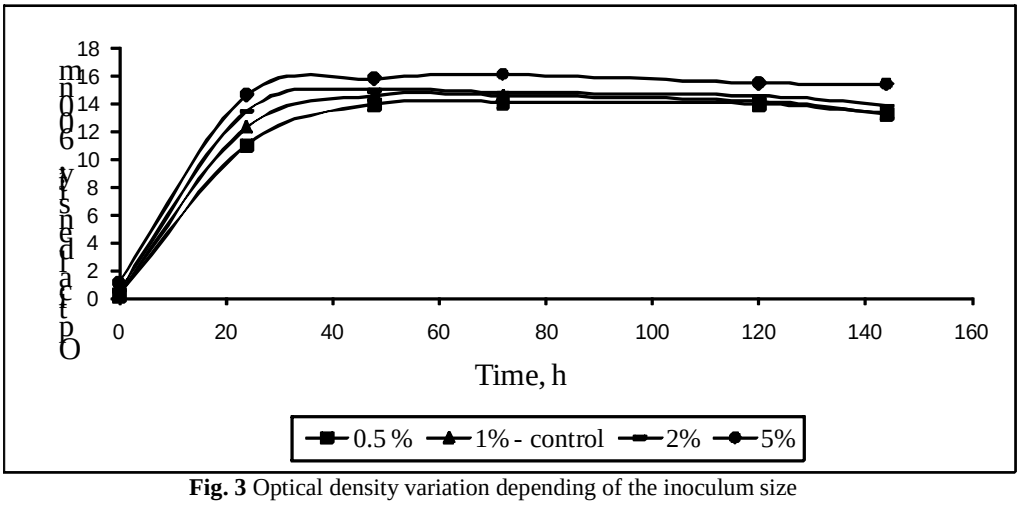

Fig. 3 Optical density variation depending of the inoculum size

An inoculum concentration of 1-2 v/v is favorable for both yeast growth and carotenoids formation including the torularhodin accumulation.

3. Study of the nitrogen source influence

In a first step, growth and carotenoids synthesis, including the torularhodin formation with the *Rhodotorula rubra* yeast were investigated based on the use of different salts as mineral nitrogen sources such as NH_4NO_3, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, NH_4Cl and NH_4NO_3 (as the control salt initially present in MS3 medium) with the concentration of 5 g/L for all the variants. At the same time for the control medium composition variant the organic nitrogen source, the yeast extract, was supplemented with a concentration of 0.1% from the following amino acids: tryptophan, threonine, glutamic acid, cysteine, alanine, tyrosine, proline, leucine and valine. The experiment lasted for 144 h.

The pigments formation results are presented in the fig. 4.

Table 1. Experimental variants notation function of the use of different nitrogen sources

Experimental variants	Nitrogen source, 5 g/L
1	NH_4NO_3 - control
2	$NH_4H_2PO_4$
3	$(NH_4)_2SO_4$
4	NH_4Cl
5	NH4NO3 + 0.1% tryptophan
6	NH4NO3 + 0.1% threonine
7	NH4NO3 + 0.1% glutamic acid
8	NH4NO3 + 0.1% cysteine
9	NH4NO3 + 0.1% alanine
10	NH4NO3 + 0.1% tyrosine
11	NH4NO3 + 0.1% proline
12	NH4NO3 + 0.1% leucine
13	NH4NO3 + 0.1% valine

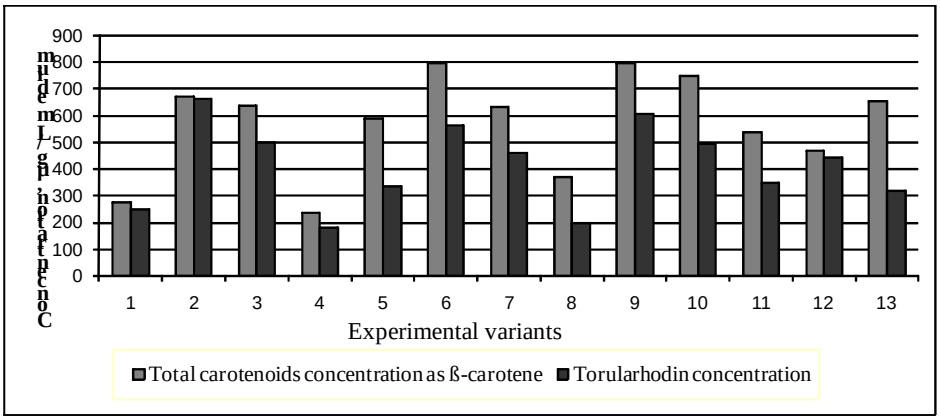

Fig. 4 Variation of the torularhodin concentration, and the total carotenoids concentration (as β-carotene) depending of the nitrogen source from the broth

Compared to control medium (MS3) all the variants characterized by small additions of amino acids have better growths. The introduction of alanine and threonine seems to

influence more the pigments formation than the use of $NH_4H_2PO_4$ instead of the control mineral nitrogen source.

4. Influence of fatty acids additions

In order to study the possible influence of the introduction of small concentrations of fatty acids on growth and pigments formation considering the ***Rhodotorula*** yeasts capacity to assimilate these acids as valuable carbon sources [11] the control medium formula was supplemented with 0.1% of fatty acids: linoleic/oleic/stearic/palmitic. Again the growth and the total carotenoids concentration compared with the torularhodin concentration were determined. The carotenoids formation, including torularhodin is presented in the Table 2, and the growth curves for the same experimental variants are represented in the fig. 5.

Table 2. Experimental variants notation function of the use of fatty acids

Medium composition variants	Fatty acids addition
C	Control (MS3)
L	Glucose + 0.1% linoleic acid
O	Glucose + 0.1% oleic acid
S	Glucose + 0.1% stearic acid
P	Glucose + 0.1% palmitic acid

The growth was better when oleic acid was used and the carotenoids and torularhodin concentrations are the highest for this variant.
It is to mention that the influence of the addition of small concentrations of amino acids alanine or threonine was more important.

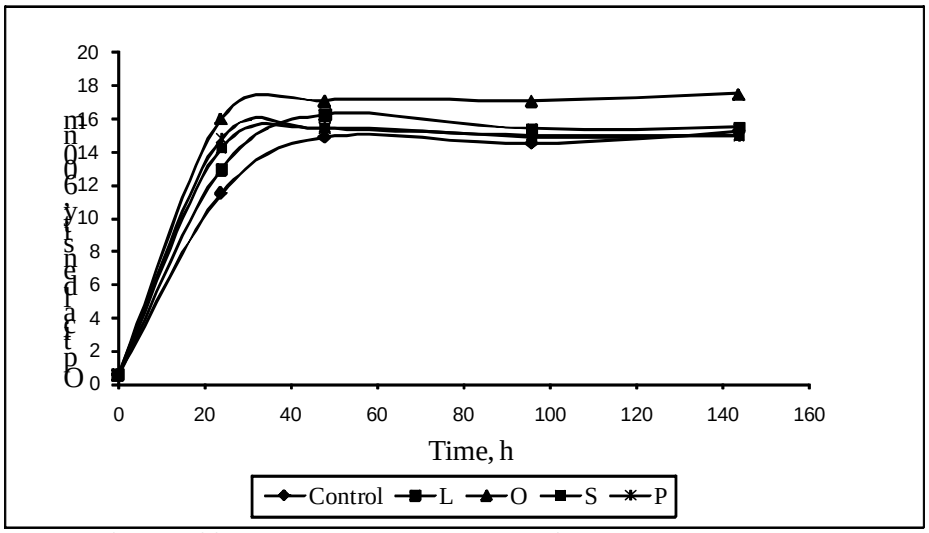

Fig. 5 Influence of fatty acids addition on the growth of *Rhodotorula rubra* ICCF 209 yeast

5. Influence of cultivation time

Several experiments were realized to determine the influence of the cultivation time, in the range 1 – 6 days, on the yeast growth and both – total carotenoids and torularhodin formation were measured. The stationary phase characterized by an important production of carotenoid pigments starts after 48 hours in all cases.

The total carotenoids concentration and the torularhodin concentration were determined based on the spectrometric recording of the extracts on the UV-VIS spectrophotometer (Jenway Spectrophotometer).

The total carotenoids concentration was determined as β-carotene content by using the extract absorbance values (fig. 6); similar the torularhodin concentration was calculated using methanol extracts absorbance values (fig. 7) with the specific absorption coefficient, $E^{1\%}1932$.

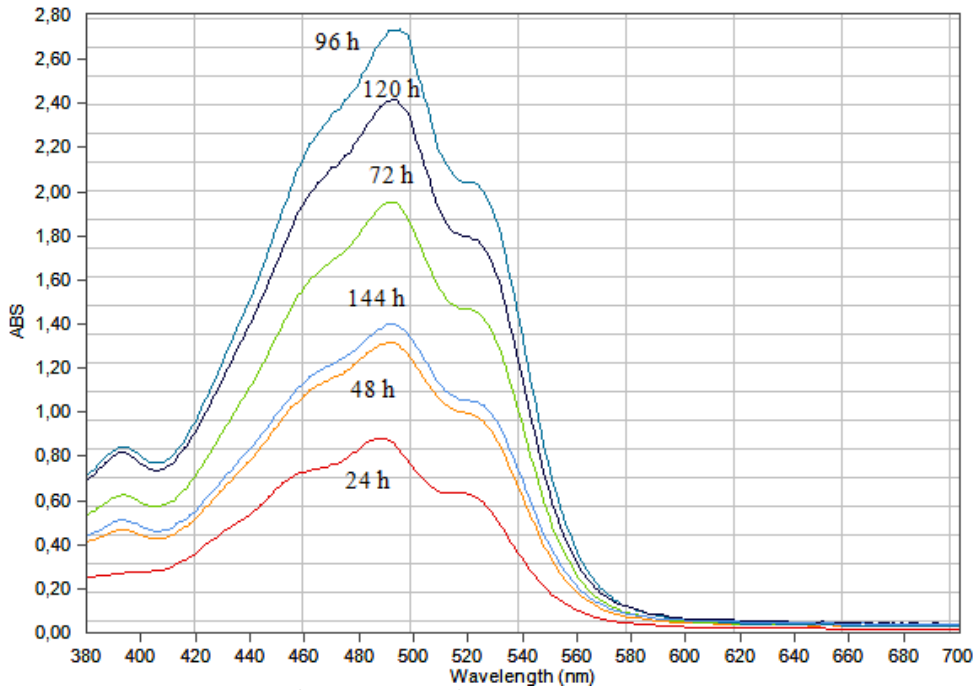

Fig. 6. Absorption spectra of hexane extract for the *Rhodotorula rubra* ICCF 209 culture in MS3

Until 48 h the ratio between the torularhodin concentration and the total carotenoids concentration is small, so the yeast biosynthesizes more the other pigments; then during the growth stationary phase the concentration of torularhodin can represent so far 90% from total carotenoids content. The carotenoids concentration increases until 96-120 h, but after this duration the trend is towards the diminution of any carotenoids content.

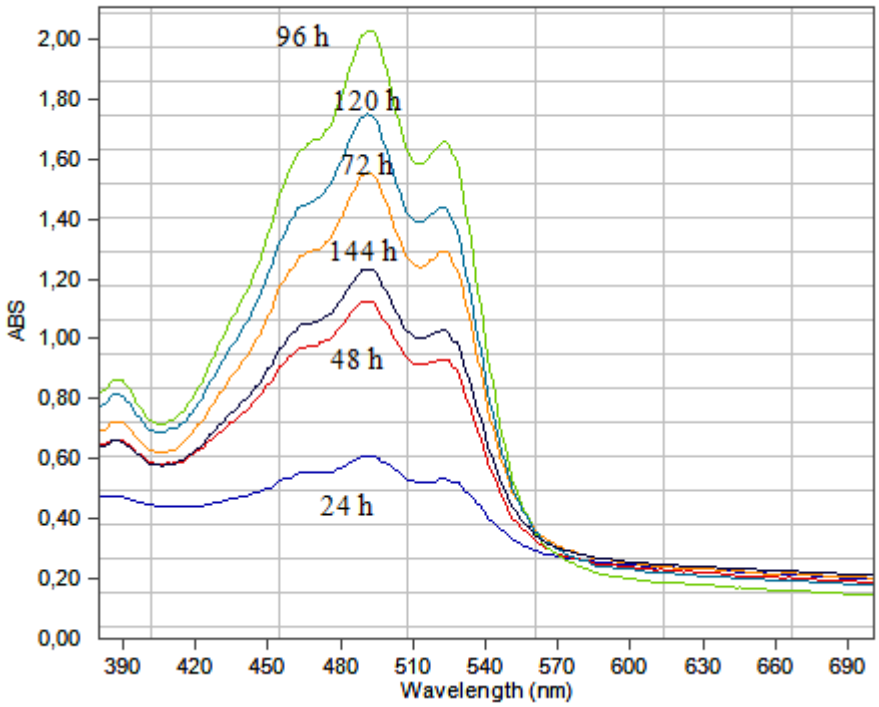

Fig. 7 Absorption spectra of methanol extracts for the *Rhodotorula rubra* ICCF 209 culture in MS3

4. CONCLUSIONS

Culture media have a considerable influence on the yeast biomass accumulation and carotenoid pigments biosynthesis, particularly torularhodin, component with a high-level antioxidant potential.

For all carbon sources tested the *Rhodotorula rubra* ICCF 209 strain presented a good growth as can be seen from the OD_{600} nm values. The yeast strain growth is stimulated by glucose, fructose, sucrose and maltose, but inhibited by lactose.

The studied characteristics – yeast growth, the total carotenoids formation, and the torularhodin formation recommend the initial pH range of 6-7 as being favorable, the torularhodin ratio from the total carotenoids content being greater for the pH of 7.

At the same time an inoculum concentration of 1-2 % is favorable for both yeast growth and carotenoids formation.

When NH_4NO_3 is replaced in the control medium with other mineral nitrogen sources ($NH_4H_2PO_4$, $(NH_4)_2SO_4$, or NH_4Cl) both the growth and the carotenoids formation are higher when acid ammonium phosphate was used; in this case a fraction of about 95 % torularhodin is formed in the pigments mixture.

The medium composition variant, where there were both - the replacement of NH_4NO_3 as anorganic nitrogen source with the same concentration of - $NH_4H_2PO_4$ and the supplementation with alanine (0.1%), threonine (0.2%), and oleic acid (0.1%) – represents the optimum composition studied so far for torularhodin formation.

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THE MATHEMATICAL MODELING IN OPTIMIZATION OF REFRIGERATION PROCESS

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Abstract. *In recent decades there is a continuing concern in the food industry for optimization of refrigeration process. Improvement of conservation technologies of perishable food products has as primary objective the combination of capabilities extending the validity of the product with keep physical, chemical, nutritional and sensory properties. The mathematical modeling is a fundamental solution to which appeals most often to optimize a random process, including the refrigeration.*
In the first part of this paper we present the main theoretical elements that characterize the optimization process using certain mathematical models. Then, we apply the theoretical concepts previously presented to optimize two refrigeration processes.

Keywords: **mathematical model, stochastic optimization, evolutionary method**

1. INTRODUCTION

It is known that refrigeration has a key role in food safety; at present there are several technologies that apply conservation of perishable food products (radiation, drying, curing, high-pressure processing and conservation). With these techniques is intended primarily to extend the combined capacity of validity of the product, while maintaining the physical, chemical, nutritional and sensory qualities, the objective being pursued in the same way by both consumers and manufacturers of refrigeration systems.

An important consequence of the widespread use of refrigeration systems is to ensure better nutrition worldwide both in terms of quantity and quality of perishable food. In terms of quality, it should be emphasized that chilling leads to reduce damage to vital food of animal and vegetable nature (which can accommodate different pathogens) and hence the decline in bacterial growth.

The above are some arguments that justify the importance accorded to refrigeration processes. Permanent concern of researchers for optimizing food refrigeration processes is so logical, although until now small steps have been taken in this direction. Tradition, certain regulations in the field, the criteria by which food or processes are structured as an optimization problem (choice of objective function and non-quantitative definition of variables subject to large errors), contributed to the development of interest in this direction of the food industry.

In recent years there have been several studies to modeling food processes that enable some products that were intangible qualities such as taste, tenderness, color, safety and other quality factors to be quantifiable and predictable.

Data processing techniques have evolved through improved observation, experiments gave errors and time consuming, and mathematical modeling has become the method most often used for optimization of refrigeration processes.

2. MATERIALS AND METHODS

We will try to apply the mathematical optimization for two refrigeration processes, in order to improve product quality and increase food safety. We used for this, the studies indicated in references with [2] and [4].

The first problem that we want to optimize applying mathematical modeling is "how to completely thaw blocks of meat in a given time while minimizing surface temperature in order to minimize microbial growth, by varying the air temperature in a suitable way ", also knowing that when the air temperature is too high, the surface will heat up and increase the number of microbes, while if it is too low the centre will not thaw in time.

The second problem that what we study is "beef carcass chilling optimization." We want to model a system of temperature, given the constraints and maximizing the tenderness of the loin muscle and reducing microbial growth rate.

Before presenting solutions, we see that we have analyzed a phenomenon occurring in certain variables (air temperature at the surface and the center block of meat) and restrictions (increasing the number of microbes to be minimal), that have the general data of a problem optimization. It is necessary to introduce some features of mathematical modeling and optimization that we subsequently apply the above mentioned problems. We used in this order [1], [3] and [7].

2.1. The mathematical modeling

The mathematical modeling has an important role in modern scientific research. In construction of a mathematical model, those characteristics of the object modeling are highlight on one hand and on the other hand for the informative characteristics we consider the mathematical formalization. The mathematical model is a mathematical relationship describing the essential properties of the original. So, solving a real problem can be reduced to solving a mathematical problem.

The general mathematical modeling steps are formulation of the problem, developing a model, computer experimentation (simulation) and analysis of modeling results (satisfactory or unsatisfactory).

Depending on the sizes involved in models, they can be deterministic (the precise size value) or stochastic (a part of all sizes take random values, for example occurs in temperature, humidity, etc.).

The stochastic modeling is a relatively new branch in mathematical programming, in which the results and methods of deterministic programming and the theory of probability are used. The stochastic programming aims to study the problems of determining the best decision possible solutions when the crowd is not completely known (in the deterministic sense) but predictable, due to the presence of random factors specified, and the objective function is a function defined on the set of random possible solutions [5]. In such problems it is considered as random factors involved in defining possible solutions crowd and the objective function (status parameters) to form a single multi-dimensional random variable, whose distribution law is unknown. An important feature of stochastic methods is that they tend to imitate natural phenomena.

There are two principles in addressing stochastic programming:
a) the principle of "wait and see" is intended that the knowledge of probabilistic information about n-dimensional random vector, and distribution function, or some moments (mean, variance), stochastic problem is then treated as a deterministic problem, characterized by a probability distribution.
b) The 'here and now "means choosing a solution from the set of possible solutions that meet a certain criterion of optimality, before studying random variables involved in the problem.

2.2. General formulation of optimization problems

The optimization process is divided in three stages:
- Problem definition, in which one define the system (process) to be optimized, the variables that can be changed, the objective function to calculate the minimum or maximum, and limitations to be observed;
- Construction of a mathematical model to be used to calculate the objective function;
- Choosing of mathematical methods for finding the minimum or maximum objective function.

2.2.1. Problem definition

The objective function is what we seek to maximize or minimize. This is usually an economic function (e.g.: energy consumption, equipment size, production costs, capital costs, total cost, the duration of the recovery of money or the internal rate of return) or non-economic (safety and reliability, product quality or dependence work or often a combination of these factors, weighted in some way reflecting our priorities). Often, the goal is to minimize costs, therefore the objective function is called the "cost".

The variables can be modeled - design variables such as number, size and structure types of equipment or plants, or operational variables such as freezers and coolers programming, how to rotate refrigeration compressors or number of laps in a day. The variables can also be classified as continuous variables (or simple) and structural variables. The former tend to be more easily optimized, while structural variables (those which cannot conveniently be made in figures) are the most difficult to represent mathematically and therefore most uncomfortable for optimization.

The constrains of optimization problems are: physical (laws of conservation of energy or materials, laws of thermodynamics), economical (capital available, the rate of return or recovery time), technological (materials and technology available), and others are legal and sociological.

Basically a mathematical optimization problem (linear or nonlinear programming) is to determinate maximum or minimum (optimum) of a function that depends on certain variables, that respect the restrictions given.

2.2.2. Construction of a mathematical model

The optimization process is performed on a mathematical model. It must establish a set of equations that expect the objective function (cost) and a set of variable values together with a method of solving these equations. The equations are generally solved by a numerical procedure.

2.2.3. An evolutionary method for process optimization

For problems involving optimization of a process where variables model can vary over time (or any other independent variables such as distance) turn to an evolutionary program [6]. For example, heat or chill food processing, efficiency and resulting product quality are dependent on the time-temperature regime. The method is based on random temperature regimes. Each of these regimes (also called members of the population) consists of a vector $X\left(X_1,...,X_n\right)$ whose components depend on the (constant) equal intervals of time $t_1,...t_n$, $t_{i+1}-t_i=t$, $\left(\forall\right)i=\overline{1,n}$, the temperature values at times are determined by the linear interpolation or using Lagrange polynomial.

Starting from two random vectors and using a mathematical formula we obtain the following vector-temperature (this step is called a generation reproductive stage). Then, two vectors are taken at random from those indicated. The "less competent" (for the refrigeration process- the highest temperature) of them is eliminated.

This process of elimination provides vectors temperature range finding "fit" for the process considered. The algorithm is repeated until it reaches a satisfactory regime existing in the population until they can get improvements for a pre-specified number of cycles.

3. RESULTS AND DISCUSSIONS

3.1. Optimize defrosting meat blocks

The problem of optimization of complete thawing meat blocks, considering the temperature fluctuation in a certain period of time, was solved by applying the evolutionary method above, after having first found the following:

- Using a conventional technique of numerical analysis, the calculus of finite differences, we can determine the temperature at different points of the food.
- If we consider the temperature variations in the block of meat to be thawed are high because the edges and corners are heated more quickly and if we consider the deviations due to random errors of measurement or recording, we believe that stochastic modeling is the most appropriate method to optimize this process.
- The meat blocks are represented by a plate 160 mm thick; initial temperature of -20 ° C, heat transfer coefficient is 20 W/m2 K and the time available for thawing 24 hours.
- We assume that the temperature varies, but because of design restrictions vaporizer chamber cannot exceed 30°C, graphical representation of this temperature is a polygonal line with a slope change every three hours.
- The objective function we want to determine is the extent to which the minimum surface temperature exceeds 7 ° C and is characterized mathematically by:

$$f=\sqrt{\int\left[\max\left(0,T_s-7\right)\right]^2},$$

Where T_S is the surface temperature.

- The cost of thawing is given by the penalty function:

$$g=\max\left(0,T_P-T_C\right)$$

Where T_C is the temperature in any point of the block of meat and T_P is the temperature in the center. We want to determine

$$\min\left(f + cg\right)$$

c is the coefficient of penalty cost.

To ensure that the packaging is completely thawed, choose a high cost factor, for example. To determine the optimal solution, it is necessary to achieve a number of different simulations (between 500 and 1000). Starting from the maximum value of 30 ° C air temperature drops to 24-23 ° C, then at 20 ° C and finally at 8 ° C. This process keeps the surface temperature to no more than about 13 ° C, while the center is thawed in 24 hours. The results obtained with the evolutionary method have the next graphical representation:

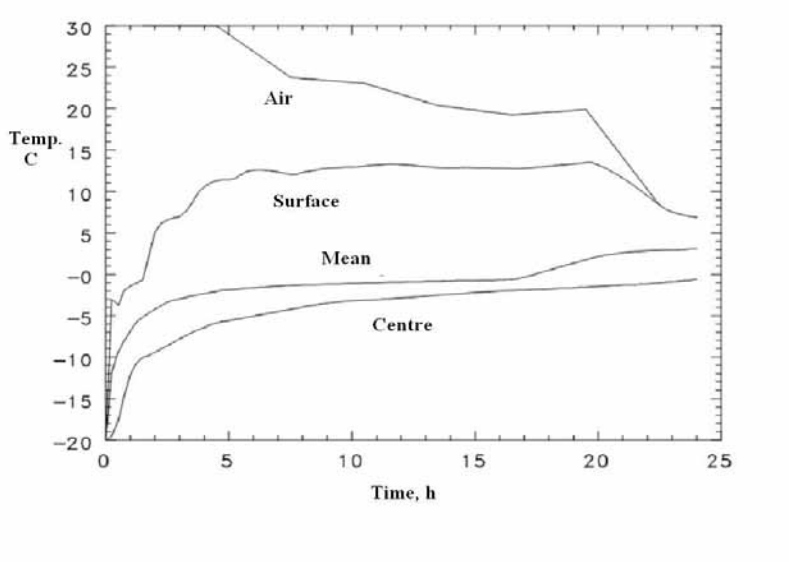

Fig.1 Optimized thawing process for meat blocks.

3.2. Optimization of beef carcass chilling (microbial constrains)

We propose to model a system to cool the temperature of a foot deep beef carcasse, such as the potential microbial (E. coli) growth on the surface of the foot should not exceed three generations and at the same time to obtain maximum tenderness of the meat. Heat transfer coefficient is assumed to be 20 W/m2K and initial temperature 40 ° C. The air temperature ranges from 15 ° C and -10 ° C because of regulations and design restrictions.

The problem was proposed and analyzed by Q. Tuan Pham and S. Lovatt [4]. The mathematical model corresponding to E.coli growth rate (Gill, 1993), indicates both necessary intervals beef carcass cooling according to different values of temperature and microbial growth rate (in E.coli generations per hour).

If t_L is the latent period (in hours), and *r* is the rate of microbial growth, there are the following relationships:

Table 1. Intervals beef carcass cooling according to different values of temperature and microbial growth rate

T	t_L	$\sqrt{r}$
$T < 7^\circ$	$t_L = 0$	$\sqrt{r}$ =0
$7^\circ \le T < 30^\circ$	$t_L = 10^{0,001T^2 - 0,136T + 2,841}$	$\sqrt{r} = 0,0513 \cdot T - 0,17$
$30^\circ \le T < 40^\circ$	$t_L = 10^{0,001T^2 - 0,136T + 2,841}$	$\sqrt{r} = 0,027 \cdot T + 0,55$
$40^\circ \le T < 45^\circ$	$t_L = 10^{0,001T^2 - 0,136T + 2,841}$	$\sqrt{r} = 2,66$
$45^\circ \le T < 47^\circ$	$t_L = \infty$	$\sqrt{r} = 2,66$
$T > 47^\circ$	$t_L = \infty$	$\sqrt{r} = 0$

The meat tenderness is measured by a z value, ranging from 1 (strong meat) before cooling to 0. Z is the standard objective function, while the penalty function is *g*. These expressions are combined as the objective function. The optimization is then carried out routinely using an evolutionary program.

The objective function and penalty function are formed in the normal manner. The air temperature is gradually reduced from 15 ° C to 0.8 ° C, then increased back to 5.3 ° C in the last hours. This system provides a nearly linear decrease in average temperature of the rear leg and the final temperature is 14 ° C. The tenderizers degree is normalized from 0252-0194 with a significant decrease of 23%.

4. CONCLUSIONS

Through this work we wanted to present in a more accessible form the general aspects of mathematical modeling and optimization techniques that are used increasingly often in the food industry. The two applications presented above are examples in this sense. In the first problem analyzed, we consider the temperature variations in the block of meat to be thawed are high because the edges and corners are heated more quickly and if we consider the random errors of measurement due to deviations or registration, we believe that stochastic modeling would be a more appropriate method to optimize this process. In the second problem studied, we wanted to model a system of temperature, given the constraints and maximizing the tenderness of the loin muscle and reducing microbial growth rate.

For both problems we could apply both methods in parallel: the evolutionary method and the stochastic procedure.

We intend in the future to apply the stochastic modeling for refrigeration processes, in order to compare the results obtained by this technique with those obtained by evolutionary method.

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STUDY METHODS TO EXTRACTION OF PHENOLIC COMPOUNDS IN THE PROCESS OF OBTAINING ROSE WINES WITH STABLE CHROMATIC CHARACTERISTICS

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Abstract. *Lately at European level is distinguished in our country and a growing demand for rosé wines, due to freshness, fruitiness and olfactory characteristics of these wines. However, to obtain quality rosé wines technologist faces numerous problems, mostly related to extraction of phenolic compounds, so the chromatic characteristics of wine taste and balance.*

Several methods have been studied for extraction of polyphenols from grape varieties typically used to obtain dark red wines: Cabernet Sauvignon, Feteasca neagra and Pinot noir: extraction of phenolic compounds by maceration of grape marc short duration (5 h, 10 h), at a temperature of 18 °C– V_1; extraction by maceration-fermentation at 20-22 ºC temperature - V_2; criomaceration pulp at a temperature of 5 ° C.

The results of physico-chemical and organoleptic results that the method of extraction of polyphenols shows a great importance for color and organoleptic characteristics of wine, each variety of black grapes with a potential technology of phenolic compounds, behaving differently depending on the version of the extraction used. Of the three grape varieties, Cabernet Sauvignon, followed by Feteasca neagra variety shows a large pool of phenolic compounds compared with Pinot noir.

Thus, for the first two varieties of the best ways of extracting them is short maceration and criomaceration; by fermentation-maceration is high concentration of phenolic compounds. The wines are intensely colored and lacking the finesse of rosé wines.

For Pinot noir, a variety of phenolic potential lower than the other two varieties is the best option fermentation-maceration, the criomaceration is the method by which to extract the small amount of phenols.

Keywords: rose wines, polyphenol extraction, criomaceration

1. INTRODUCTION

In recent years, our country and especially the rest of Europe there is an increasing demand for rosé wines, especially in summer. To obtain high quality rose wines using grape varieties for red wines and follows a portion of total polyphenols extraction to achieve balanced wine, smooth, light-colored, with personality.

Of course, many rosé wines on the market are fakes, that is, wines produced with different methods: by passing a white wine by marc result from getting red wines, white wine mixed with red wine, both different in origin.

Due to increasing demand, many countries in Europe (France, Italy) develops new technologies for obtaining quality rosé wines, varietal specific technologies and specific conditions. In some vineyards in Romania rose wines are produced by various methods and technologies (direct fermentation of musts made from grape varieties poorly colored, rose wines obtained from mixed technology).

2. MATERIALS AND METHODS

The establishment of technological schemes for obtaining quality rosé wines used black grape varieties that are commonly used to make red wines: *Cabernet Sauvignon, Feteasca neagra* and *Pinot noir*. The grapes were vinified by three different technological schemes (variants V_1, V_2, V_3):

V_1: short maceration of grapes (5-10 h) at temperature of 18ºC;

V_2: maceration-fermentation at temperature of 20-22º C;

V3: criomaceration, temperature of 5 º C, for *Pinot noir*.

Resulting wines were analyzed by the following parameters: alcoholic strength (vol% alcohol), total acidity (g/l tartaric acid), volatile acidity (mg/l CH_3COOH), dry extract (g/l), phenolic compounds (mg/100 ml), anthocyan content (mg/l), intensity and shade color.

Grapes harvested from the three varieties have slightly different concentrations of sugars, a higher concentration with *Pinot Noir*, the alcoholic strength of wine is also higher for this variety. Sugar content was 218 g/l to *Pinot Noir*, 215 g/l to *Feteasca neagra* and 206 g/l for *Cabernet Sauvignon*.

3. RESULTS AND DISCUSSIONS

Table 1 – Chemical and physical parameters of rose wines obtained by two types of extraction of polyphenols

Chemical and physical parameters of rose wines/ Parametrii fizico-chimici ai vinurilor	*Feteasca neagra*		*Pinot noir*		*Cabernet Sauvignon*	
	V_1	**V_2**	**V_1**	**V_2**	**V_1**	**V_2**
alcoholic strength, vol% alcohol	12,5	12,6	12,7	12,7	11,9	12,0
residual sugar, g/l	2,5	0,8	2,2	2,2	3,7	2,0
total acidity, g/l tartaric acid	5,5	5,4	5,1	5,1	5,9	5,9
dry extract, g/l	28	34	28	32	32	38
volatile acidity, mg/l CH_3COOH	0,3	0,5	0,3	0,5	0,4	0,3
phenolic compounds, mg/100 ml	38,8	47,49	32,0	38,82	42,5	58,5
anthocyan content, mg/l	214,98	242,31	185,3	205,2	225,4	262,1
color intensity DO520 nm + DO420 nm	0,638	0,659	0,624	0,635	0,720	0,79
shade color DO520 nm /DO420 nm	0,65	0,69	0,54	0,64	0,79	0,82
scoring wines to taste	I	III	II	I	II	III

Color extraction process steps to obtain rose wines are of special importance, for taking the time and temperature used depends on the characteristics resulting wine. Thus, each black grape variety with a different technological potential of phenolic compounds, behave differently in different types of color extraction. Wines produced by variant V_2, maceration-fermentation at temperature 20-22º C, has an increased content of phenolic compounds from V_1. In the case of *Cabernet Sauvignon* wine, polyphenol concentration is higher by 37,6 % over the first variant (short extraction at 18° C). In the *Feteasca neagra* wine polyphenols concentration increased by 22,3 % in variant 2 compared to variant 1,

and for *Pinot noir* wine, one can observe an increase of 21,3 %. Of the three grape varieties, *Cabernet Sauvignon*, followed by *Feteasca neagra* has a large pool of phenolic compounds compared to *Pinot Noir*.

And in anthocyan content extraction by V_2 leads to increased concentrations of these compounds color: 16,2 % to *Cabernet*, 12,7 % to *Feteasca neagra* and 10,7 % to *Pinot noir*. The content of phenolic compounds reduced the *Pinot Noir* can be observed for anthocyanins.

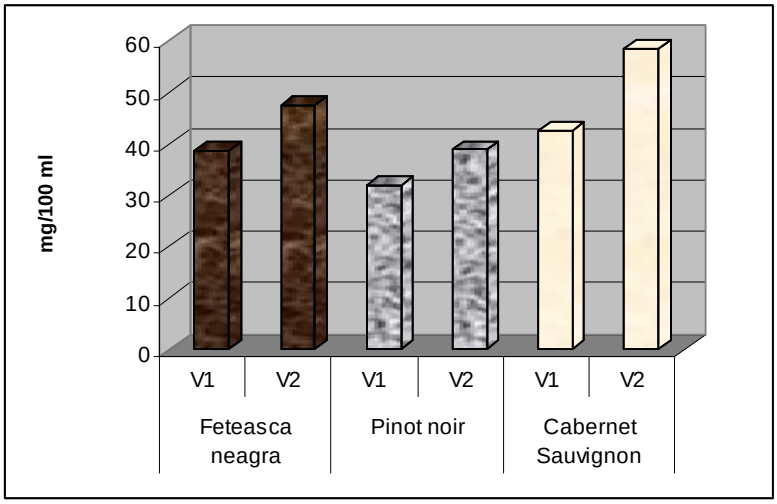

Fig.1 Variant influence over the content in polyphenols extraction

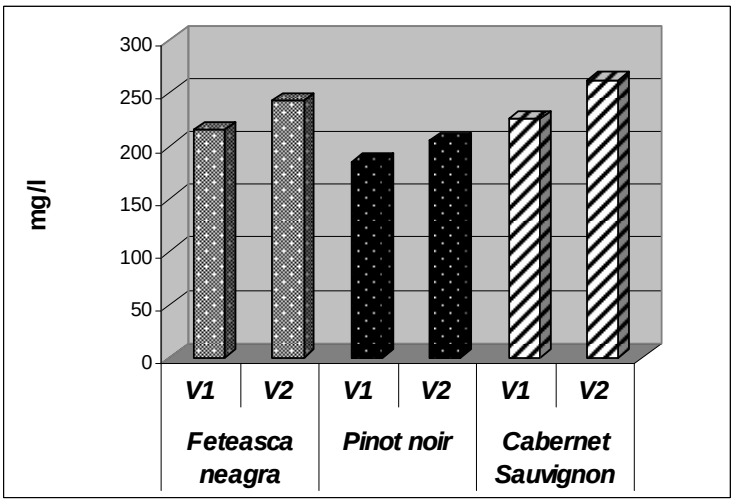

Fig.2 Variant influence over the content of anthocyanin extraction

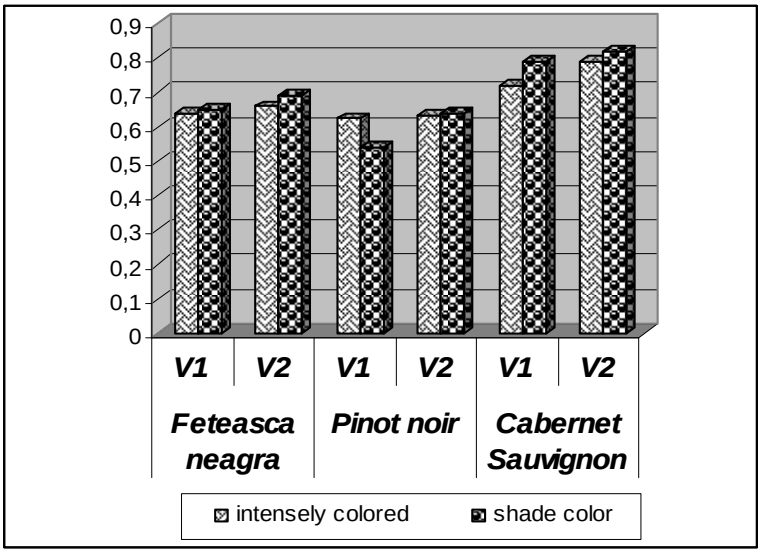

Fig. 3 Variant influence on the intensity and shade color wines

In *Pinot Noir*, phenolic compounds content is lower and thus is more suitable for obtaining rose wines, the main problem in obtaining this wine is not to get too rich color and taste tannins as in red wines. In *Pinot Noir* should be used for extraction version V_2, resulting wines fits best in class quality rose wine, balanced, with optimal content of polyphenols, fresh and pleasant aroma. The color is pink, medium intensity.

Besides the chemical analysis of wines was made and tasting organoleptic, the results led to the conclusion that to obtain rose wines of *Cabernet Sauvignon* may be used only version of short maceration of grapes (5 h) at temperature of 18º C and for obtaining the *Feteasca neagra* rose wines can apply the same technology but with a longer duration of extraction (10 h).

Table 2. Influence of criomaceration on wine quality *Pinot Noir*

Chemical and physical parameters of rose wines/ Parametrii fizico-chimici ai vinurilor	**V_1**	**V_3**
alcoholic strength, vol% alcohol	12,7	12,8
residual sugar, g/l	2,2	0,4
total acidity, g/l tartaric acid	5,1	5,0
volatile acidity, mg/l CH_3COOH	0,5	0,2
phenolic compounds, mg/100 ml	37,05	26,65
anthocyan content, mg/l	206,9	163,6
color intensity DO520 nm + DO420 nm	0,912	0,531
shade color DO520 nm /DO420 nm	I	III

For *Pinot Noir* has been studied a criomaceration method, extraction of polyphenols at low temperature, the extraction method leads to obtain fresh wine of great finesse. Witness is classical maceration at temperature 18 ºC.

In *Pinot Noir*, this method has yielded good results, extraction of phenolic compounds and anthocyanins were insufficient. Staining intensity of the wines that had very small values compared with controls.

4. CONCLUSIONS

1. The method of extraction of polyphenols from rose wines is a major technological step, temperature used depending on the quality of wines;

2. Each black grape variety has a different technological potential of phenolic compounds and behaves differently from color extraction;

3. To obtain rose wines of *Cabernet Sauvignon* may be used only the version of short maceration of grapes (5 h) at temperature of 18º C and for obtaining the *Feteasca neagra* rose wines can apply the same technology but with a longer duration of extraction (10 h).

4. In *Pinot Noir* should be used for extraction version V_2, resulting wines fits best in the class of quality rose wines, balanced, with optimal content of polyphenols, fresh and pleasant aroma. The color is pink, with medium intensity;

5. In *Pinot Noir*, this method has yielded good results, extraction of phenolic compounds and anthocyanins were insufficient. Staining intensity of the wines that had very small values compared with controls.

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STUDY OF FACTORS EVENT INFLUENCING THE QUALITY OF RED WINES

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Abstract. *The quality wine is a result of the interdependence of many factors, a very important role is attributed to the ecosystem of the vineyard and the climatic conditions of the harvest.*
Mode of recovery of environmental conditions (soil - local climate and microclimate - climatic factors of year of harvest) is done by every variety of vine in the metabolism of specific. It was studied the influence of environmental conditions on the technological potential and quality of wines from Cabernet Sauvignon and Merlot in 3 wine centers: Dragasani, Valea Calugareasca and Urlati, during a 3 year interval (2007 – 2009).
The results of experiments showed that both vineyard ecosystem and climate of the harvest has a major influence on the technological potential of the variety that, the quality of wine results. Significant differences were recorded on color characteristics of grapes and wines of three varieties analyzed.
In terms of year of harvest, 2007 saw the most favorable climatic conditions, this positive influence both production and quality of wines in both viticulture centers analyzed. Merlot varieties fared best in the center Valea Calugareasca and wine center Urlati offered the best conditions of grape variety Cabernet Sauvignon.

Keywords: ecoclimatic factors, grape varieties, red wine

1. INTRODUCTION

Grape variety is always in close interdependence with ecological and geographical conditions, with its specific biotope and ecosystem. Also, crop quality and therefore the quality of wine results vary in a given ecosystem to the climate of the year. Mode of recovery of environmental conditions is achieved by the metabolism of each specific variety. Studies on the system variety-ecosystem-crop year have great importance for establishing each type of varieties of a viticultural area date in order to obtain the best production, particularly in terms of quality. Climate change in recent years requires similar studies for ecosystem restoration in the range of varieties Romanian wine.

2. MATERIALS AND METHODS

To establish the influence of the ecosystem vineyard and the harvest year were studied two varieties of black grapes, *Cabernet Sauvignon* and *Merlot*, which are varieties that occupy the largest area in Romanian vineyards, to obtain high quality red wines. Vineyard ecosystems studied are three wine-growing areas in southern Romania, specialized in obtaining quality red wines: Dragasani (EC I); Valea Calugareasca (EC II) and Urlati (EC III). Studies were conducted in 2007-2009.

Were analyzed several parameters of quality grapes and wines: sugars accumulated, (g/l sugars), total acidity (g/l tartaric acid), production (t/ha); alcoholic

strength (vol% ethilic alcohol), wine total acidity (g/l tartaric acid), volatile acidity (mg/l CH_3COOH), dry extract (g/l), phenolic compounds (mg/100 ml), anthocyan content (mg/l). Tests were performed according to standards in force.

3. RESULTS AND DISCUSSIONS

Observations and analysis showed the influence of ecosystem vineyard and harvest year on the quantity and quality of grapes and wines. Thus, the *Cabernet Sauvignon* was greater accumulation of sugars in the center Urlati wine, sugar concentration in the three centers registered in values between 205 and 230 g/l; in terms of harvest year there were large differences, as with ecosystems, 2007 the best year, so the accumulation of sugars largest. 2007 was considered the best year in all wine areas in the south and across the country.

Merlot showed differences in quality and quantity depending on the vineyard and the vintage year. Favorable climatic conditions of 2007 were positively influenced the accumulation of sugars and balance of taste (sugar/acidity). In terms of vineyard area, although the analysis focused on the same center vineyards, *Merlot* was better in Valea Calugareasca wine center, an interesting factor is equal accumulation of sugars in 2007 and 2008.

In the case of wines, different accumulation of sugars led to different alcohol concentrations, but perfectly in agreement with the values of this parameter, so the *Cabernet* wine is highest in the alcoholic strength in Urlati center in 2007 (13,3 vol% alcohol) and a lower alcoholic strength, but not in disagreement with the potential variety in the Dragasani area. Total dry extract good values recorded in all years and in all three growing centers. Chromatic compounds accumulation is done in parallel and in a positive relationship with the accumulation of sugars. Often, high concentrations of phenolic compounds characteristic of the variety is reached before a high concentration of sugars, but only under a year when climatic factors have allowed.

Table 1 – Technological potential of *Cabernet Sauvignon* and *Merlot* vines in three ecosystems in the period 2007-2009

Chemical and physical parameters of grapes/ Parametrii fizico-chimici ai strugurilor	*total acidity, g/l tartaric acid*		*grape production t/ha*	
	Cabernet Sauvignon	*Merlot*	*Cabernet Sauvignon*	*Merlot*
vineyard ecosystem I, 2007	6,85	6,9	15,9	19,0
vineyard ecosystem I, 2008	6,85	7,19	17,4	20,2
vineyard ecosystem I, 2009	7,50	7,15	17,0	20,0
vineyard ecosystem II, 2007	7,25	7,2	16,0	17,8
vineyard ecosystem II, 2008	7,65	7,5	15,5	17,5
vineyard ecosystem II, 2009	7,89	7,58	15,5	18,3
vineyard ecosystem III, 2007	6,90	6,45	13,1	17,5
vineyard ecosystem III, 2008	7,14	7,0	14,0	18,0
vineyard ecosystem III, 2009	7,70	7,15	14,8	18,3

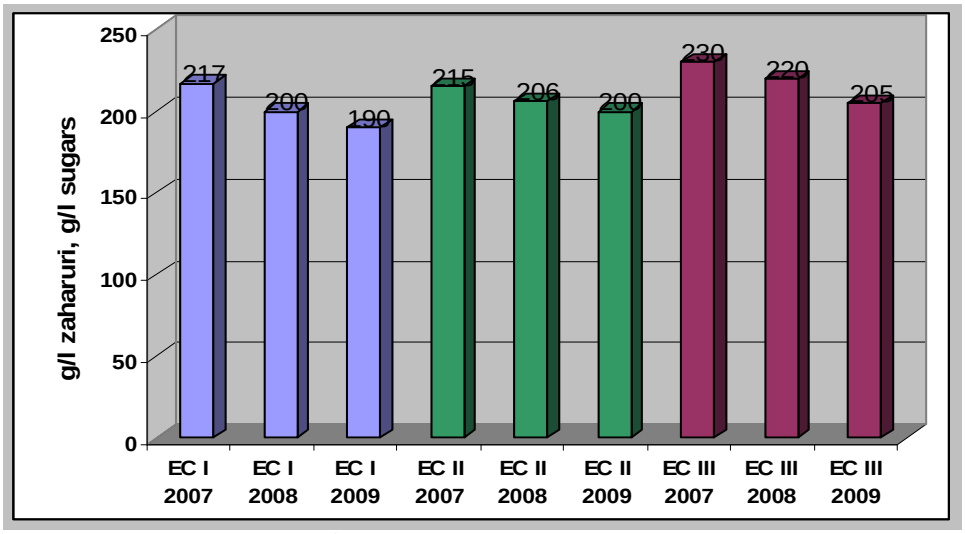

Fig. 1 Variation accumulation of sugars in *Cabernet Sauvignon* in three vineyard ecosystems in 2007-2009

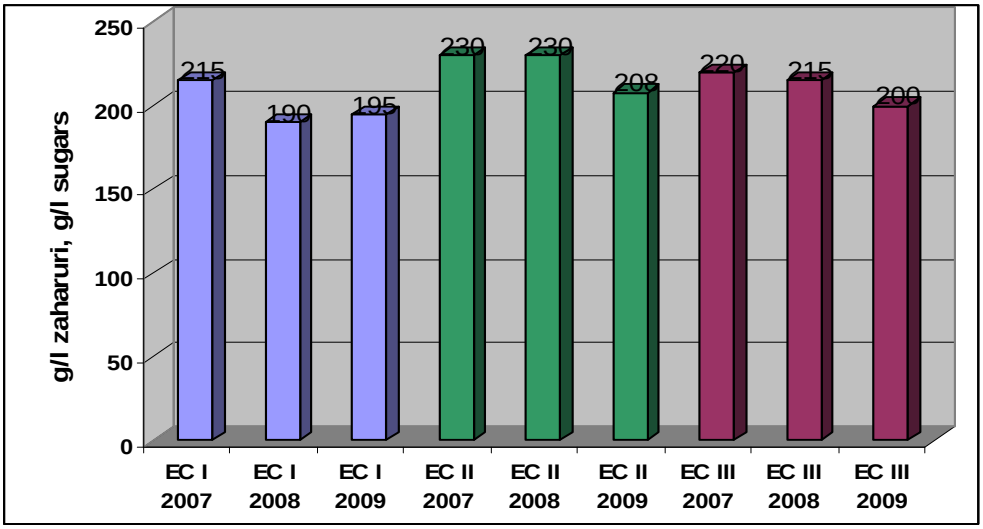

Fig. 2 Variation accumulation of sugars in *Merlot* in three vineyard ecosystems in 2007-2009

Analyses showed that in the case of *Cabernet Sauvignon* wine, there were still large amounts of phenolic compounds in wine center Urlati in 2007. Tannins content varied very little a larger amount of tannins occurring in Urlati, amid higher concentrations in phenolic compounds. In anthocyanin concentration followed the same curve, higher

accumulation occurring in Urlati center, the difference is quite large between the center and center Dragasani.

Merlot wine analyzed showed values of key parameters in total agreement with the compositional quality of the grapes: the values of the alcoholic strength of acidity (total and volatile), extract and color characteristics are higher in the center of Valea Calugareasca; values in the other two centers were different and less non-characteristic, and in 2007 was still one that has influenced this case the best quality wines.

Table 2 – Quality parameters of wine *Cabernet Sauvignon*

Quality parameters of wine / Parametrii de calitate ai vinurilor	alcoholic strength, vol% alcohol /taria alcoolica	total acidity, g/l tartaric acid/aciditate totala	dry extract g/l /extract sec	volatile acidity, mg/l CH_3COOH/ aciditatea volatila
vineyard ecosystem I, 2007	12,0	5,4	26,4	0,4
vineyard ecosystem I, 2008	11,6	5,6	26,2	0,2
vineyard ecosystem I, 2009	11,0	6,1	26,0	0,3
vineyard ecosystem II, 2007	12,4	6,1	32,0	0,3
vineyard ecosystem II, 2008	12,0	6,5	31,7	0,3
vineyard ecosystem II, 2009	11,7	6,7	30,3	0,4
vineyard ecosystem III, 2007	13,3	5,8	33,0	0,4
vineyard ecosystem III, 2008	12,7	6,0	31,7	0,3
vineyard ecosystem III, 2009	12,0	6,5	30,5	0,5

Table 3 - Quality parameters of wine *Merlot*

Quality parameters of wine / Parametrii de calitate ai vinurilor	alcoholic strength, vol% alcohol /taria alcoolica	total acidity, g/l tartaric acid/aciditate totala	dry extract g/l /extract sec	alcoholic strength, vol% alcohol /taria alcoolica
vineyard ecosystem I, 2007	12,4	5,8	25,5	0,4
vineyard ecosystem I, 2008	11,0	6,0	24,0	0,4
vineyard ecosystem I, 2009	11,0	6,1	24,8	0,3
vineyard ecosystem II, 2007	13,2	5,2	30,3	0,4
vineyard ecosystem II, 2008	13,0	5,3	30,2	0,2
vineyard ecosystem II, 2009	12,1	5,5	30,0	0,4
vineyard ecosystem III, 2007	12,7	5,1	27,0	0,5
vineyard ecosystem III, 2008	12,4	5,5	27,3	0,3
vineyard ecosystem III, 2009	11,3	5,9	25,0	0,3

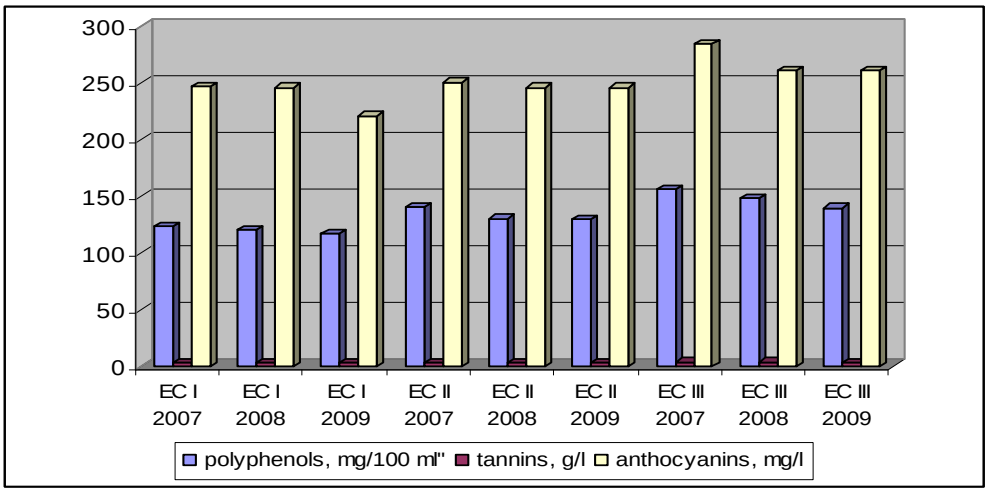

Fig. 3 Variation of content in phenolic compounds, tannins and anthocyanins at *Cabernet Sauvignon*

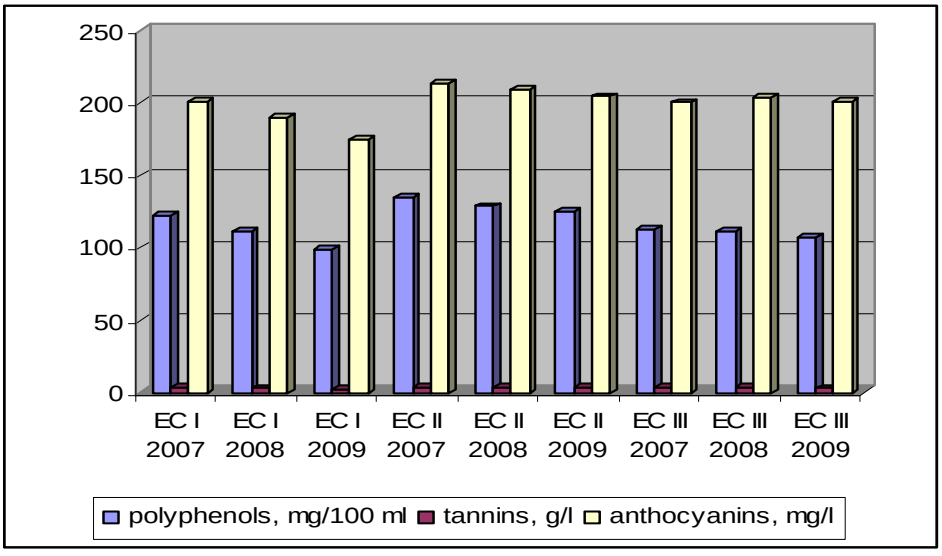

Fig. 4 Variation of content in phenolic compounds, tannins and anthocyanins at *Merlot*

4. CONCLUSIONS

1. The quality of grapes and wines is a result of a multifactorial system, wine variety is always lying in close interdependence with ecological and geographical conditions, with its specific biotope and ecosystem;

2. The crop quality in a given ecosystem vary with climatic conditions of the year (soil- local climate-climate year);

3. The analyzed varieties, *Cabernet Sauvignon* and *Merlot* have behaved differently, depending on environmental conditions and growing zone but depending on climatic conditions of the crop year.

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SECTION IV: INDUSTRIAL AND ENVIRONMENTAL BIOTECHNOLOGY

MOLASSES INFLUENCE ON THE EFFICIENCY OF INDUSTRIAL BIOTECHNOLOGICAL PROCESSES OF PRODUCING YEAST BIOMASS

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Abstract. *The aim of this study was to investigate the behaviour of selected yeast strains of Saccharomyces cerevisiae to produce baking yeast biomass on different substrates. The molasses represents the main raw material for baking yeast industry in Romania and the most important role for an efficient baking yeast technological process is the quality of the molasses used as raw material. The main objective of the research was to investigate the differences in yeast biomass accumulation between sugar beet and sugar cane molasses supplemented with biotin, inositol and pantothenic acid. The yield in biomass was calculated for both categories of molasses and a comparison between technological variants was performed in order to settle the most economical variant for the culture medium. The technological variant using sugar beet molasses used as raw material supplemented with biotin registered comparable results with the variant using sugar beet molasses in mixture with sugar cane molasses.*
The results of this study suggest the feasibility of cheaper and more efficient technological procedure that can be applied in the industrial biotechnological processes.

Keywords: molasses, baking yeast, nutrients, process efficiency

1. INTRODUCTION

Many different carbohydrate feedstocks can be used for yeast production, but because of low costs most companies use cane or beet molasses. Molasses are dense and viscous liquids, with a high amount of solid residues (68–75%) and 48–52% of sucrose [Burrows, 1970]. The molasses quality can affect the yield and quality of the yeast produced because its composition is highly variable depending on the sucrose reffining procedure and on the specific location, soil type, production process and year's weather conditions [Kristiansen, 1994: Maqueda et al., 2011].

Nowadays, molasses is the standard growth medium for baking yeast and is the cheapest source of carbohydrate [Goksungur and Zorlu, 2001], but many years passed before it replaced saccharified cereals. The substitution of saccharified cereal mash by

molasses improved yeast yield, even in the absence of aeration. Historically speaking, Claudon and Vigreux appeared to have been the first to patent in 1882 a process describing the use of molasses for growing yeast and in 1895 Elion proposed to use molasses enriched with phosphoric acid. The early twentieth century, large amounts of molasses became available at minimal price for yeast manufacturing [Gelinas, 2010].

In the last four years European Union registered a decline in sugar beet production after the limitation of exports by the World Trade Organization. [http://www.agroinfo.ro, 2011]. As regards the sugar beet situation in Romania, in this moment the area cultivated is around 20000 ha and it is sure that sugar beet will be considered in the next future as an energy crop. The cultivated area has halved after Romanian accession (from 40,000 hectares in 2006) when it was established in negotiations with the EU a quota of sugar for Romania [http://www.capital.ro, 2011]. Under these conditions, alcohol and yeast factories in Romania were forced to resort to imports of sugar cane molasses from countries such as Brazil.

Currently applied production technologies for baking yeast provide continuous accumulation of biomass. The main purpose of manufacturing technology of baking yeast is to obtain a quantity of high quality yeast biomass with minimum consumption of raw materials and utilities. The modern baking yeast manufacturing was established around 1920. [Gelinas, 2010]

Saccharomyces cerevisiae known as baking yeast can be considered one of the most cultivated microorganism, and has been used extensively for the production of single-cell protein (SCP) used as food and feed. Baking yeast belongs to the species *Saccharomyces cerevisiae*, according to the classification of Hansen in 1904, and represents only a yeast culture [Hansen, 1896]. Baking yeast is top fermenting yeast strain capable of producing fermentation of sugars in the dough with the formation of ethyl alcohol and CO_2, raising agents of dough, and other products, with a role in bread-making. One of the most important requisites in the commercial production of baker's yeast is rapid growth and high biomass yield [Benitez et al., 1996].

As regards the utilisation of adjuncts in order to increase technological yields, as early as 1877 specific supplements for liquid media (such as nitrogen and vitamins) were used. Starting with 1883, molasses supplementation with nitrogen sources as ammonium salts was proposed by Scard for promoting yeast growth at alcohol production, a practice that would later be very important in modern yeast manufacturing [Gelinas, 2010]. Nitrogen is normally supplied through the addition of ammonium salts, aqueous ammonia, or anhydrous ammonia [Chen and Chiger, 1985].

Mineral requirements of molasses include nitrogen, potassium, phosphate, magnesium, and calcium. As regards potassium and calcium, molasses normally provides sufficient quantities of these minerals. Phosphates and magnesium are added in the form of phosphoric acid or phosphate salts and magnesium salts [Reed and Peppler, 1973]. Besides these, molasses needs iron, zinc, copper, manganese, and molybdenum are also required in trace amounts.

The yeast growth process also requires several vitamins, such as biotin, inositol, pantothenic acid, and thiamine. In the absence of biotin baking yeast is not able to grow. Both cane and beet molasses usually provide enough inositol and pantothenic acid for yeast growth in order to obtain maximum yields of biomass. Beet molasses is deficient in biotin

and the variations of this factor explain in most of the times, in practice, variable yields of yeast [Reed and Peppler, 1973; Assessment of VOC emissions, 1992; Manualul inginerului, 2002; White, 1954].

The present study is in line with other researches in order to provide the most appropriate technological solutions as procedures to be applied in the industrial biotechnological processes, such as food fermentation industries. The aim of this study was to investigate the comparison in yeast biomass accumulation between culture media based on sugar beet and sugar cane molasses, in order to propose the most suitable formulation under specific conditions of decline of sugar industry in Romania.

2. MATERIALS AND METHODS

ANALYTICAL METHODS

- The soluble dry matter content in molasses was determined by AOAC Official Method 932.14, using a double dilution of molasses;
- The moisture content in molasses was determined by AOAC Official Method 966.20 (Karl Fisher method);
- The total sugar, initial reducing sugar and sucrose in molasses were determined by Luff-Schoorl method;
- The protein content in pressed biomass was determined by Kjeldahl method, calculated as N x 6.25 (AOAC 920.53), using 0.5 g test portion (AOAC 962.10). Digestion was performed for 30 min after solution cleared. The protein was determined on cell biomass harvested at the end of the experiments;
- The ash content in molasses was determined by AOAC Official Method 900.02 (gravimetric method, using 5 g test portion). Result was expressed as percent ash and the ash content was calculated by using the formula given below:
  Ash (g/l00g) = [(weight of dish with sample after calcination - weight of empty dish/weight of dish with sample before calcination - weight of empty dish) x 0.9] x 1000.9 – coefficient for transformation of sulphated ash into conventional ash;
- The solid content in yeast biomass was determined by AOAC Official Method 961.06 (the method of 16 hours drying);
- The yeast cell concentration was estimated by measuring spectrophotometrically in UV-Vis the optical density (OD) of the culture at 600 nm using a conversion factor of 0.50 OD for 1 $\times 10^7$ cells, and also by weighing the dried biomass samples on aluminium dishes at 100°C for 24 h;
- Life yeast cells (viability) were measured by methylene blue stain.

EXPERIMENTAL ORGANISMS (MICRO-ORGANISMS AND INOCULUM)

A pure culture of *S. cerevisiae* (ICA Collection of Microorganisms) was used throughout this work for the formation of yeast biomass. The yeast strains used in this work *S. cerevisiae* baking yeasts MY2011 previously isolated and selected in order to obtain baking yeast biomass [Regodon et al., 1997].

MAINTENANCE MEDIUM

The maintenance medium was YPG agar. The yeasts were maintained on a slant of YPGA medium (yeast extract 1%, peptone 1%, glucose 2%, and agar 2% (w/v)) and stored at 4°C.

INOCULUM

The culture medium was portioned in 1000 ml Erlenmeyer flasks, which were cotton-plugged and sterilized at 121°C for 15 min. When the flasks attained room temperature, they were inoculated (2% v/v) with the yeasts. The culture medium for inoculum was malt-agar (malt extract 3%, peptone 0.5%, agar 2%).

The yeast were cultivated in 1000 ml Erlenmeyer flasks containing 500 ml of medium at 30^0C on a Unimax 1010 orbital rotatory shaker with Incubator 1000 (Heidolph) at 150 rpm for 24 h. Microaerobic conditions were provided by the use of flasks with cottonwool plugs. At the end of the fermentation process, the final pH and the yeast biomasses were determined.

The inoculum (10% of the working volume and roughly 5.2 x 10^7 cells/ml) was transferred from the flasks to stainless steel bioreactor (total volume of 20 l) fitted with pH monitoring and controlling equipment and dosage equipment (raw material, nutrient salt solutions) and which contained approx. 15 l liquid volume (work was performed below 75% of bioreactor capacity to avoid foam problems).

FERMENTATION MEDIA

The preparation of molasses used as fermentation medium comprises the following groups of technological operations: dilution of molasses with potable water at a concentration of 40% d.m.; acidification of molasses using concentrated sulphuric acid (pH final 4.5 – 4.8); sterilisation of molasses; clarification of diluted and sterilized molasses through decantation and filtration.

The molasses tested were characterized through the following parameters:

- Sugar beet molasses - soluble dry matter 80.63%; moisture content 22.58%; total sugar 57.71% expressed in glucose; initial reducing sugar 9.25%; sucrose 50.48%; ash 5.7%; pH 5.7 (in solution 10%); nitrogen content 1.23%;
- Sugar cane molasses - soluble dry matter 82.2%; moisture content 23.7%; total sugar 59.24% expressed in glucose; initial reducing sugar 11.3%; sucrose 55%; ash 7.8%; pH 6 (in solution 10%); nitrogen content 1.19%.

The minerals necessary for yeast were introduced into the culture medium in the form of solutions.

The yeast multiplication was tested, with and without addition of vitamins (biotin, calcium panthotenat and inositol) to the medium based on molasses. The vitamins were added one by one and as mixture of two or three within the culture medium.

The soluble dry matter content for the molasses starting base medium before inoculation in the bioreactor was 20^0Brix.

The media based on molasses contained the following ingredients (g/l):

Medium I: $(NH4)_2S0_4$, 0.6; KH_2PO_4, 0.3; in 1000ml distilled water;

Medium II: $MgSO_4x7H_2O$, 0.3; $CuSO_4x7H_2O$, 0.002; $ZnSO_4x7H_2O$, 0.02; $CaCl_2x2H_2O$, 0.08; $(NH4)_2S0_4$, 0.6; KH_2PO_4, 0.3; in 1000ml distilled water;

Medium III: $MgSO_4x7H_2O$, 0.3; $CuSO_4x7H_2O$, 0.002; $ZnSO_4x7H_2O$, 0.02; $CaCl_2x2H_2O$, 0.08; $(NH4)_2S0_4$, 0.6; KH_2PO_4, 0.3; inositol, 0.23; calcium panthotenat, 0.05; in 1000ml distilled water;

Medium IV: $MgSO_4x7H_2O$, 0.3; $CuSO_4x7H_2O$, 0.002; $ZnSO_4x7H_2O$, 0.02; $CaCl_2x2H_2O$, 0.08; $(NH4)_2S0_4$, 0.6; KH_2PO_4, 0.3; inositol, 0.23; calcium panthotenat, 0.05; biotin, $0.06x10^{-3}$; in 1000ml distilled water.

Formulations were tested, combining in different rations the sugar beet and sugar cane molasses. The initial pH values of all media were adjusted to 4.5 with H_2SO_4 conc.

FERMENTATION CONDITIONS

Before inoculation, the culture medium in the bioreactor was steam sterilized *in situ* and after cooling was inoculated with 10% inoculum (24-hours-old).

The molasses was fed-batch added following a program, maintaining the sugar concentration around 8%, in order to avoid sugar fermentation and ethanol production. The feeding diagram with molasses is presented in fig. 1.

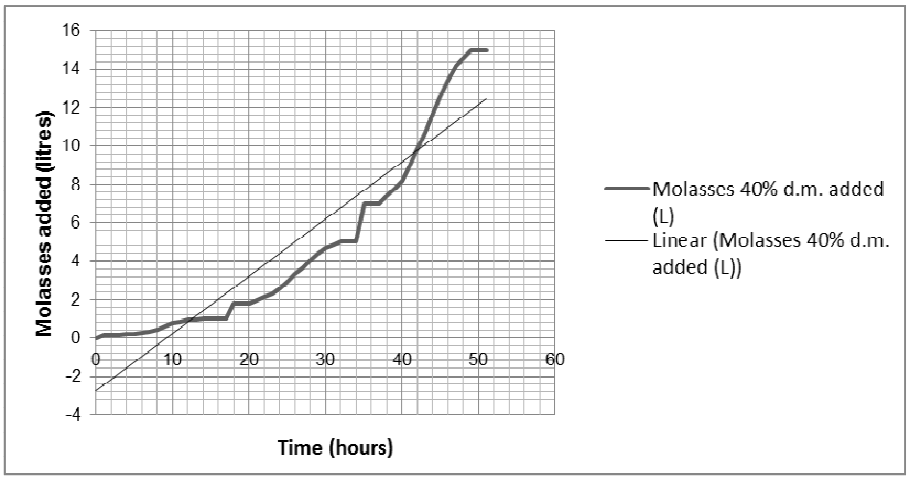

Fig. 1 Molasses addition programs for baking yeast production

Fermentation was performed at a temperature of 30^0C. Agitation speed and aeration rate in the bioreactor were 1000 rpm and 0.6 litres / litres / min, respectively. The pH was automatically controlled at pH 4.5-4.8 with 2N NaOH. Silicone antifoam (Carl Roth) was used to prevent foam formation. The total fermentation time was 51 hours, and after completion of the molasses addition, the culture was maintained with aeration for 4h to allow yeast maturation.

Parameters such air flow, oxygen concentration in the culture, sugars and ammonia in the medium are controlled in commercial yeast production to improve yield [Kristiansen, 1994; Lee et al., 1999; Maqueda et al., 2011], but these parameters were not monitored during this work.

At the end of multiplication process (55 hours) the biomass was separated by centrifugation using a laboratory centrifuge ROTINA 38 Hettich with 250 ml tubes. The yeast biomass was centrifuged for 20 minutes at 4000 rpm. In order to assess the yield in biomass, the amount of biomass formed after 55 hours of fermentation was determined. Cell growth was measured by determining the optical density at 600 nm using a spectrophotometer. To determine culture dry weight, culture samples (10 ml) were filtered

through pre-weighed 13-mm membrane filters (Nalgene, 0.45-µm pore size). The filters were washed with distilled water twice and then dried at 100°C for at least 16 h to constant weight. The determinations were performed in duplicate and varied by less than 2%.

3. RESULTS AND DISCUSSIONS

Microscopy revealed that most of the yeasts produced were pure culture, with homogeneous cell size and morphology for molasses productions (Fig. 2), with more than 98% of live cells as measured by methylene blue stain.

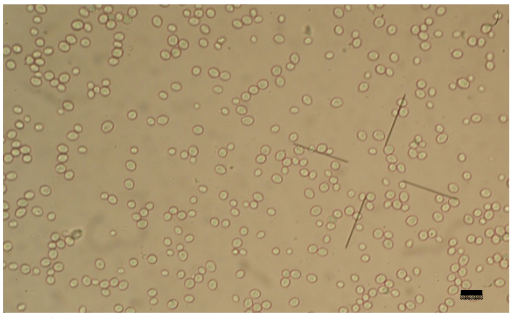

Fig. 2 Cell shape of strains for MY2011 yeast produced in the 20-l bioreactor (16 l of culture) with molasses. The micrographs were taken after the 24-h production run and the *scale bar* represents 10 µm.

YEAST YIELD FOR THE FED-BATCH MOLASSES PRODUCTION

The yield of yeast production with molasses depended on the amount of total sugar existing during yeast growth. In figures 3 and 4 is given the yield in biomass (YB, in grams of dry yeast/l) for the tested formulations of culture medium: sugar beet molasses (SBM), sugar cane molasses (SCM) and mixtures (half ratio) of sugar beet and sugar cane molasses (SB+C_M). Also the presence of certain nutrients into the medium growth contributes to different biomass accumulation (enrichment for). In this work we used four various medium, as it was mentioned above.

As is shown in figure 3, the biomass yield increased from a minimum of 24.9 g/l using blank medium (I) for sugar beet molasses to a maximum value of 33.3 g/l, when medium IV and sugar cane molasses were used for.

Figures registered throughout our study indicated that the best yield was obtained using sugar cane molasses, no matters the media preparation. Obviously, the results were improved when they worked with formulation of medium IV in comparison with previous ones due to specific enrichments.

On the basis of the data introduced in figures 3 and 4, the following remarks can be drawn on either the influence of type of molasses or ingredients for fermentation preparation on the biomass accumulation in culture medium:

1. For all tests there is an increment in biomass yield with an upward trend of percentage of the cane molasses in the culture medium (see also figure 5).

2. Increase in yield of biomass in the case of medium II in comparison with medium III is similar. This is in accordance with literature [Reed and Peppler, 1973; Assessment of VOC emissions, 1992] in that the cane and beet molasses usually provide enough inositol and pantothenic acid for yeast growth.

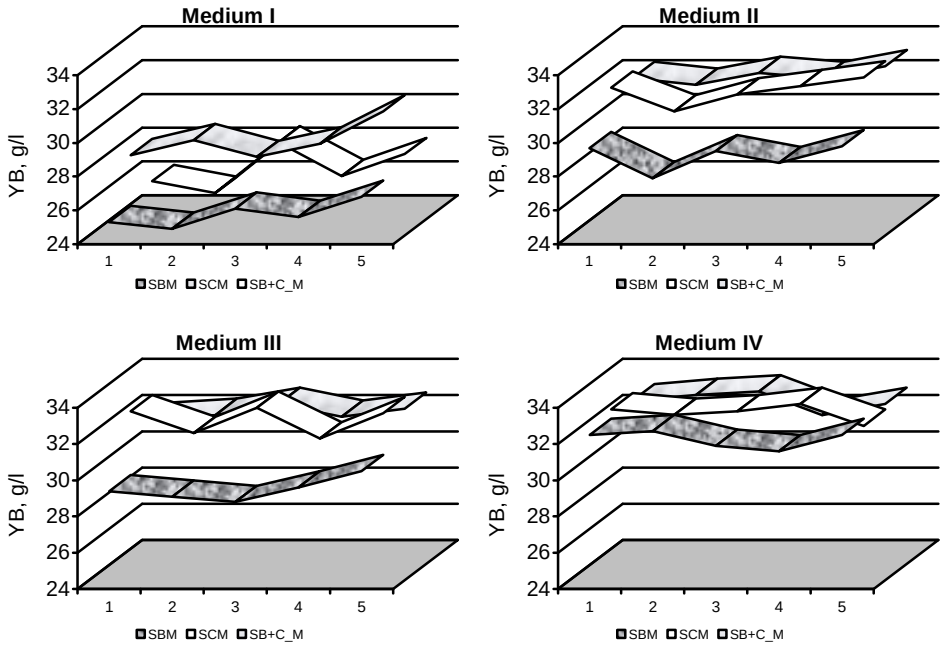

Fig. 3 Variation of the field in biomass depending on growth medium composition

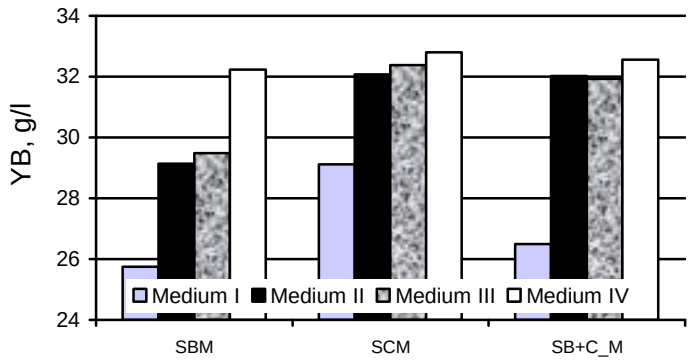

Fig. 4 Mean values for yeast yield production by using different fermentation media

3. Differences on biomass growth were register between medium II and medium IV when various molasses used, but bivariate correlation coefficient was not significant at p=0.05.

4. The results performed on medium III for sugar cane molasses are similar with results for medium IV for sugar beet molasses, which is in accordance with [Reed and Peppler, 1973; Assessment of VOC emissions, 1992] findings that sugar cane molasses provide in some extent biotin as growth factor for yeast growth, compared with sugar beet molasses; similar outcomes are noticed for the medium IV using sugar beet molasses with addition of biotin (32.24 g/l) and medium II using a mixture of sugar cane and sugar beet molasses (32.02 g/l).

In other words, our results highline findings [Reed and Peppler, 1973; Assessment of VOC emissions, 1992] that sugar beet molasses is deficient in biotin and the variations of this factor explain in practice in most of the times the variable yields of yeast. Anyway the addition of biotin, in all cases, leads to an increase amount of yield in biomass in the culture medium.

5. In the case of both types of molasses the largest quantity of biomass is obtained when all three growth factors are present; but biotin seems to have a decisive role as regards the amount of biomass produced at the end of the multiplication.

6. The yeast yields for all media tested were reasonably well as compared with other yeast biomass productions. In this respect, according to our results data, the yield was similar with other type of yeast biomass, such as those produced with an ingredients mix of molasses and cheese whey, 32.3–35 g/l [Ferrari et al., 2001].

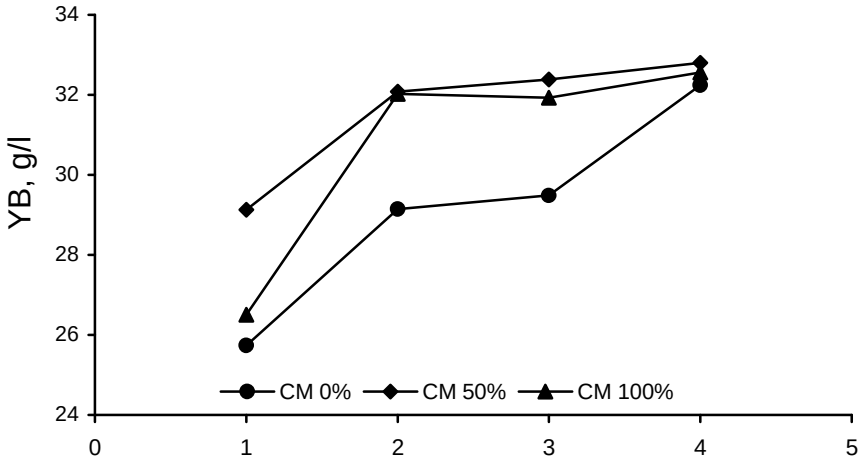

Fig. 5 Yeast yield production in relationship with cane molasses (CM) content

4. CONCLUSIONS

The addition of growth factors, such as microelements and other ingredients contributes to improvement of technological yield in yeast biomass.

The data generally showed that MY2011 yeast registered not significant difference in biomass production when the number of supplements increased. Yeast cultivated in

medium based on sugar beet molasses supplemented with mineral salts and vitamins responded in a similar way to medium based on mixture of molasses supplemented with minerals. Given that the incorporation of many ingredients requires more input into the industrial production of yeast biomass, the formulation of media based on mixture of molasses with incorporation of ammonium salts and minerals is considered as cost-effective in condition.

The most significant influence on the yield in biomass is represented by biotin. The results of this study indicate the feasibility of industrial application of two solutions, in order to obtain a similar yield in yeast biomass:

→ utilization of sugar beet molasses as main raw material, supplemented with biotin;

→ utilization of a mixture of sugar beet and sugar cane molasses, with no addition of vitamins.

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IN VITRO CULTURES OF BALKAN AND BULGARIAN ENDEMIC *CENTAUREA* SPECIES

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Abstract. *Centaurea caliacrae and C. finazzeri are Balkan endemic species and C. davidovii is endemic for Bulgaria. In this study, in vitro cultures of the three species were initiated from seeds as a means of ex situ conservation. Due to the species' conservation status, the available plant material was limited, especially with C. davidovii. An average of 90% of the seeds of C. caliacrae germinated on MS nutrient medium in the first 20 days of cultivation regardless of phytohormone treatment (giberellic acid). Eleven MS-based media were used with different combinations and concentrations of cytokinins and auxins. Green callus formation and occasional organogenesis were the most frequent response to plant growth regulators. A few adventitious shoots were formed by cultivated stems, especially on the medium supplemented with 0,5 g/l benzylaminopurine. On the MS medium, C. caliacrae plants grew well, rooted and were successfully transferred to ex vitro conditions with a survival rate of 84%. A single C. davidovii seedling on phytohormone-free MS medium formed adventitious stems and a callus-like tissue at the base and originated a clone, suitable for further experiments with uniform plant material.On the medium supplemented with 3,5 mg/l thidiazuron and 1,4 mg/l benzylaminopurine, leaf explants formed new leaf clusters from callus which continued growing when transferred to the MS medium.*

Keywords: knapweed, ex situ conservation, plant growth regulators

1. INTRODUCTION

The genus *Centaurea* s. l. is represented in the Bulgarian flora by more than 70 species and is among the plant groups whose center of speciation includes the territory of Bulgaria. It is also the genus richest in endemics in the Bulgarian flora (ca. 30%). In the new edition of the Red Data Book of Bulgaria (plants) (2011), 14 species are included (ca. 23%). Some of the *Centaurea* species are rare and are represented by one or few populations. This suggests measures for their conservation to be taken, both ***in situ*** and ***ex situ***, especially considering the reproductive problems those species encounter. Their seeds have a generally low germination rate and are very often damaged by insects before being dispersed. In this study, ***in vitro*** culture of three endemic species was initiated as a method for ***ex situ*** conservation – ***Centaurea caliacrae*** Prodán, ***Centaurea davidovii*** Urumov and ***Centaurea finazzeri*** Adamovič. ***C. caliacrae*** is a Balkan endemic, occurring along the Black sea coast. ***C. davidovii*** is a Bulgarian endemic, occurring only in Stara planina mountain, particularly in the Central Balkan national park. Its populations are decreasing in size (Bancheva & Gorgorov 2010). ***C. finazzeri*** is also a Balkan endemic, represented in Bulgaria by only one population in the Valley of Struma River (Northern part). In addition to ***in situ*** conservation measures, such as establishment of protected territories and habitat conservation, ***ex situ*** methods are also advisable, including ***in vitro*** cultivation and micropropagation of the

concerned plants. The *in vitro* cultivation of these three species has not been reported previously.

2. MATERIAL AND METHODS

Seeds of the three species - *Centaurea caliacrae, C. davidovii and C. finazzeri* - collected from their natural populations were used as starting material. Due to the conservation status of the species, only a small number of seeds was used – 72 of *C. caliacrae*, 45 of *C. finazzeri* and only 9 of *C. davidovii*. Thirty-four of *C. caliacrae* seeds were soaked in a 0,35% solution of giberellic acid (GA_3) for 24 hours before sterilization, and the other 38 were soaked in water. All seeds were surface sterilized by consecutive rinsing with 70% ethanol for one minute, a solution of NaClO (undiluted commercial bleach, Cl < 5%) for 10 minutes, and sterile distilled water. Then they were placed in vials containing MS nutrient medium (Murashige & Skoog 1962) and sealed with plastic caps. Until the appearance of a root tip, the seeds were kept in darkness. All following cultures were placed under a photoperiod of 16/8 h, light intensity of 2000 lux and temperature of 23-25 ºC.

For the subcultivation of the seedlings, 11 MS-based nutrient media were used, supplemented with different combinations of plant growth regulators (PGRs), with 30 g/l sucrose and 6,5 g/l Plant agar (Duchefa®) in plastic containers (Table 1). In addition to the solid media, a liquid CC1 medium in flasks on a shaker (at 100 rpm) in darkness was used in order to test if the callus would grow better in it and if somatic embryogenesis would take place. The pH of all media was adjusted to 5,7-5,8 and they were sterilized by autoclaving at 121ºC.

Plants obtained from in vitro seedlings were used as source of secondary explants: leaf and root pieces, as well as whole plants with removed roots.

For the *ex vitro* adaptation of rooted plants, a mixture of soil, coconut fiber and sand in proportion 2:1:1 was used.

Table 1. Plant growth regulators in the nutrient media (BAP: benzylaminopurine; NAA: α-naphthaleneacetic acid; 2,4-D: 2,4-dichlorophenoxyacetic acid; TDZ: thidiazuron).

Nutrient medium	Plant growth regulators [mg/l]			
	BAP	NAA	2,4-D	TDZ
MS				
C1	0.5	2.0		
C2	0.5			
C3		2.0		
C4	1.0	0.1		
C5	1.0	0.3		
C6	4.0	0.1		
C7	1.4			3.5
C8	1.5	2.5		6.0
CC1	0.5	0.5		
CC2	0.5		0.1	
CG			10	

3. RESULTS AND DISCUSSION

Centaurea caliacrae

The first *C. caliacrae* seeds to germinate were observed at the third day after being placed in the MS medium. A week thereafter half of the seeds germinated and on the 20th day, an average of 90% of the seeds germinated. The treatment with GA_3 increased the germination rate by only a few percents – 94% of the treated and 87% of the untreated seeds germinated. On the MS medium, the seedlings developed well and grew normal leaves and roots.

Secondary explants obtained from them were transferred to the different media. Green callus appeared within the first week the leaf and root explants in the PGR-supplemented media C1, C2, C3, C6, CC1 and CC2. Besides that callus, a single leaf explant on the C2 medium formed a spherical white mass of cells, and a single leaf explant on the C3 medium formed a long root. The best-looking calluses were obtained on media with relatively low concentration of both cytokinins and auxins: CC1 and CC2 (Fig. 1). Single or a few explants in the first three media also formed small leaves or roots, but they appeared vitrified and did not grow further, neither on the same media, nor on the MS medium. The whole plants always grew callus in the cut place, however it was significantly smaller in the absence of PGRs. The plants rooted best in the media with no cytokinin (C3 and MS) – 17 of 18 plants altogether, but in the media containing BAP (C1 and C2) only 2 of 18 plants formed roots. The plants on the PGR-free medium had smaller calluses and long roots.

New stems were formed on the C1, C2 and C3 media; the C2 medium had the largest number of plants with adventitious stems and up to 3 stems per plant were formed, which may be explained with the medium being supplemented with BAP only. On a medium with the same concentration of cytokinin, Cuenca & al. (1999) have obtained an average of 4,8 new shoots per explant working with *Centaurea paui* Loscos ex Willk, a species endemic for Spain. The growth of adventitious stems was observed also in 5 plants on the MS medium after they were transferred there from C2 (Fig. 2). These were 28% of the plants transferred to MS from C2, however, they did not form any roots after one month of cultivation. The formation of new shoots with good quality, capable of producing plants, was only observed with whole stem explants and not with leaf and root explants.

On a medium with the same composition as C1, Hammatt & Evans (1985) working with *Centaurea junoniana* Svent. also obtained hard green callus from leaf explants, and in that case shoot organogenesis took place in most explants and resulted in up to 7 shoots per explant. Although these authors also suggest small shoots to be transferred to PGR-free medium for elongation, *Centaurea caliacrae* leaf-derived shoots did not grow further in that medium, possibly due to vitrification.

Because of the tendency of callus-derived plants to differ genetically from the explant source, the induction of embryogenic callus was attempted using initially a variation of the medium, proposed by Robinson & al. (2009) for *Emilia zeylanica* C. B. Clarke, an Asteracean species. In our experiment, the medium was supplemented with NAA instead of 2,4-D (the CC1 medium). Some of the calluses obtained in the above media were transferred to liquid CC1 medium. After a month, the leaf- and stem-derived calluses formed yellowish-green rounded tissue clusters; their inner tissue was green and hard. The

root-derived calluses also developed rounded tissue clusters, but they were grey and less visible. The spherical cluster of white tissue, formed on a leaf explants in the C2 medium, degenerated.

Fig. 1. Callus of *C. caliacrae* on the CC2 medium (magnification 20x).

Fig. 2. A *C. caliacrae* plant with two stems.

Some leaf-derived calluses on the solid C6 medium, which has a high BAP concentration, formed clusters of new stems which had no signs of vitrification (Fig. 3). In a medium with the same composition, Wildi & al. (1998) obtained an average of 10 shoots per leaf explant in ***Petasites hybridus*** Gaertn., Mey. & Scherb.

On the CG medium, calluses did form from leaf explants, but they soon turned brown and died. The unusually high concentration of 2,4-D in that medium was expected to stimulate somatic embryogenesis by a stress-induced mechanism, but it apparently had a toxic effect instead.

Eighteen plants rooted in the MS medium were transferred to *ex vitro* conditions. They were placed in the soil mixture and were covered with transparent plastic foil for the first week. The survival rate was high – 84%, and the plants were placed in a greenhouse, where they flowered (Fig. 4).

Fig. 3. Calluses of *C. caliacrae* forming new leaves on the C6 medium.

Fig. 4. Flowering C. caliacrae plants after *ex vitro* adaptation.

Centaurea davidovii

Only one seed of *C. davidovii* germinated two months after being placed in the MS medium. Interestingly, after two more months the plant had two stems with large leaves and some long roots growing from a common base of enlarged, callus-like tissue (Fig. 5). This happened in the absence of PGRs or any other treatment. The two stems were separated and transferred to fresh MS medium, where they continued growing and forming new leaves. One of the plants grew one more stem. The callus-like tissue had numerous small leaves and was easy to divide into small clusters - 66 altogether, which were transferred to fresh MS medium. Eleven of them (16%) grew large leaves (Fig. 6).

Fig. 5. *C. davidovii* on the MS medium

Fig. 6. Tissue clusters of *C. davidoivii* growing leaves and roots on the MS medium.

Leaf and root explants from the 3 stems were placed on C4, C5, C6, C7, C8, CC1, CC2 and CG media. Those on the C4, C5 and C6 media formed calluses, but they turned brown in 6 weeks and no organogenesis was observed.

After two months on the C7 and C8 media, the leaf explants became very enlarged and formed callus, and on C7, small leaflets formed at the margins of the explants, possibly by somatic embryogenesis. Kumar & al. (2008) reported somatic embryogenesis in *Carthamus tinctorius* L in media containing 3,5 mg/l TDZ and 1,4 mg/l 6-[γ,γ dimethylallyl amino]-purine (2iP) or 6 mg/l TDZ, 1,5 mg/l 2iP and 2,5 mg/l indole-3-butyric acid (IBA). Our media C7 and C8 had the same concentration of PGRs, however we used BAP and NAA instead of 2iP and IBA respectively. Fifty-six excised leaflet clusters were transferred to MS medium, where 27 of them (48%) grew further and formed new leaves (Fig. 7).

Callus was also formed on the CC1 and CC2 media. As with *C. caliacrae*, the explants on the CG medium formed callus, but it turned brown and died.

In the five months since germination, 5 new single stems and the above-mentioned 11 clumps with large leaves were obtained from one seedling. This clone of well-propagating plants will be used for further experiments with varying media composition.

Centaurea finazzeri

Of 45 seeds, 9 (20%) germinated in the first month. None of them were treated with GA_3. After these seedlings grow, their subcultivation is intended to the media in which the other two species had the best response.

Fig. 7. Small leaflets of *C. davidovii* formed on a leaf explant on the C7 medium (left) and growing explants transferred to the MS medium (right).

Table 2. Response of the explants to the different media (C - callus; R - roots; IS - indirect shoot formation; DS - direct shoot formation).

Species	***Centaurea caliacrae***			***Centaurea davidovii***	
Type of explant / Meidum	**Leaf segments**	**Root segments**	**Whole plantlets**	**Leaf segments**	**Root segments**
MS	C, DS	C, IS	C, R		
C1	C, R, DS	C, IS	C		
C2	C	C, R	C		
C3	C, R	R, DS	C, R		
C4				C	
C5				C	
C6	C, IS			C	
C7				C, IS	C, IS
C8				C, IS	
CC1	C			C	
CC2	C			C	
CG	C (necrosis)			C (necrosis)	

4. CONCLUSION

Successful *in vitro* germination of seeds of three endemic species – *Centaurea caliacrae*, *C. davidovii* and *C. finazzeri* – was demonstrated. The germination rate of *C. caliacrae* seeds was high regardless of treatment with giberellin. The plants grew well *in vitro* on the phytohormone-free MS medium and could be used as an explant source for further experiments, as well as for *ex vitro* adaptation, which proved to be easy, with a high survival rate. The PGR-supplemented media used stimulated mainly callus formation and some shoot growth, especially when using the medium containing 0,5 mg/l BAP. The

formation of a callus-like tissue in an untreated seedling of *C. davidovii* was observed along with strong stem and root growth and formation of adventitious stems. The further collection of seeds will make it possible to clarify the frequency of this event and will provide plant material for future research.

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EPIFLUORESCENCE MICROSCOPY AND TEM INVESTIGATIONS ON BACTERIA IN MARINE MICROCOSMS

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Abstract. *In microcosms supplemented with gasoline and gasoline-enriched marine populations has developed a microbiota able to metabolize nutrients and store carbon and energy in the form of poly-β-hydroxybutyrate granules (PHB). In nalidixic acid and yeast extract medium the highest level of viable cells was observed in microcosms supplemented with gasoline (1%) and gasoline-enriched marine populations (M4). Measurements were performed using TEM analysis and automatic measurement software digital imaging, such as ImageJ to measure cell length and CellC. In microcosm 4 we detected cells (PHB) after stained with Nile blue, implying that under limited nitrogen and in the presence of source carbon, some bacteria can accumulate PHB. In epifluorescence microscopy PHB granules exhibited an orange fluorescence after staining with Nile blue by blue filter. By TEM technique we followed cell size distribution and morphology of the samples at the beginning and end of incubation natural samples. Our results on the development cell dimensions showed a temporal variation in individual size of bacterial populations in the experimental microcosms.*

Keywords: microcosms, gasoline, TEM, PHB, Nile blue

1. INTRODUCTION

It is recognized that many microorganisms, distributed in different environments, are able to use hydrocarbons as the sole source of energy and carbon (Atlas, 1981; Inoue & Horikoshi, 1991; Head & Swannell, 1999; Lazăr et al., 1999; van Hamme et al., 2003; Voicu et al., 2003; De Oteyza et al., 2004; Harayama et al., 2004; Head et al., 2006; Segura et al., 2007; Ştefănescu et al., 2008; Nikolopoulou & Kalogerakis, 2009).
The aim of this paper is to count planctonic marine prokaryotes that are able to oxidize / tolerate gasoline, to follow the dynamics of cell density over time by applying epifluorescence techniques and the algorithms of CellC and Image J software for digital images, and to investigate some ultrastructural details of these cells.

2. MATERIALS AND METHODS

Samples and microcosms

Water samples are from the Black Sea (0.5m depth), which was used for the microcosms setup done in Polyethylene transparent bottles. In our experiments, the microcosms in volume 1L of natural sea water without sediment, were kept at 18ºC temperature and fluorescent light for two months (from December 2 to January 24). Seawater microcosm was filtered through 0.45 µm to avoid the presence of protists, including heterotrophic nanoflagellates (Vazquez-Dominguez et al., 2005).

The microcosms are as follows: Black Sea natural sample- control (M3); control supplemented with gasoline (1% v/w) (M2), control supplemented with gasoline (1% v/w) and nutrients (ammonium nitrate 0.005% w/w) (M1), control supplemented with gasoline (1% v/w) and selected population (1 mL) (M4) and control supplemented with gasoline (1% v/w), nutrients (ammonium nitrate 0.005% w/w) and selected population – 1 mL (M5). Sampling was done sequentially at 1, 6, 11, 18, 39 and 53 days from the start of the experiment.

Direct viable counts

The method of direct enumeration of viable cells has followed the original protocol (Kogure et al., 1979), technique as adapted by Ghiţă et al., 2010. The seawater samples (40 mL) containing nutrients (yeast extract, 50 mg / L final concentration) and antibiotic (nalidixic acid, 20 mg / L final concentration) were kept at a constant temperature (18°C) incubated in light. Subsequently samples were harvested each two hours (considering the time T_o, T_1 –after 2 hours, T_2- after 4 hours; T_3- after 6 hours, T_4- after 8 hours, T_5- after 10 hours, T_6- after 24 hours). Then samples were stained with acridine orange (5µg/mL) and visualized by epifluorescence microscopy.

Epifluorescence microscopy

Samples were viewed immediately by epifluorescence microscopy (N-400FL, lamp Hg 100 W, type on the blue filter- 450-480 nm) with immersion 100X objective and 10X eyepieces.

Transmission electron microscopy using negative staining

To determine cell size at the beginning (T_o) and at the end of the experiment (T_6) incubation of samples in the presence of inhibitor and nutrients in the five microcosms, we used the TEM technique by negative staining with 1% acid fosfotungstic (pH 6.8-7.4), measuring 150 cells. Negative staining electron microscopy is used in one of the most widely used methods in the study of microorganisms. Contact time between biological preparation and dye (1% acid fosfotungstic) is 15 seconds.

Samples of microcosm 4 were processed and analyzed by Jastrow method (Gundersen et al., 1988; Jastrow et al., 1997) to see some adjustments in the ultrastructure of prokaryotic cell elongation from cultured cells in the presence of yeast extract and nalidixic acid. Capsules that include biological preparation are pyramid sectioned and subjected to cutting process ultramicrotom (Leica Ultracut R) to a sectional dimension of 600 Å. Observation grids was made on microscope CM Phillips 120ST.

Nile blue stain for PHB

Samples from microcosms were used for making smears which were subsequently dried with thermal fixing and stained with Nile blue solution 1% at 55^{o}C for 10 minutes (Ostle & Holt, 1982).

The automatic cell analysis was performed using the Image J and CellC software.

Fluorescence microscopy digital images were analyzed and the object has different intensity than the background. The CellC automatic quantification program presents a set of files where stored processing data are. Generally removes all detected objects that are smaller than 1 / 10 the average size of all objects (Selinummi, 2008). Also, we used Image J software to measure cell length and to obtain statistical values with the size in pixels, selected by user.

3. RESULTS AND DISCUSSION

In tabel 1 there are presended the results concerning size distribution of cells incubated 24 hours in the presence of nalidixic acid (inhibitor of DNA gyrase in Gram-negative bacteria) and yeast extract.

Table 1. The average length of cells obtained from measurement of 150 cells by TEM and epifluorescence (EFM) technique; samples were taken at the beginning of the incubation period (T_0) and at 24 hours (T_6).

incubation time	average length of cells - µm (TEM)				
	M1	M2	M3	M4	M5
T_o	0.590	0.800	0.610	0.900	0.830
T_6, 24 h	1.400	2.250	1.150	4.750	2.900
incubation time	average length of cells - µm (EFM)				
	M1	M2	M3	M4	M5
T_o	0.729	1.012	0.803	1.121	1.105
T_6, 24 h	2.597	4.264	1.996	7.888	4.118

One can see that there are some differences obtained with respect to cell length measured by epifluorescence microscopy and by transmission electron microscopy. Figure 1 and 2 are showing the correlation between the average length of cells at initial time (T_0) and final time (T_6) on direct assessment of the number of viable cells, obtained by TEM- negative staining and epifluorescence technique/ software Image J.

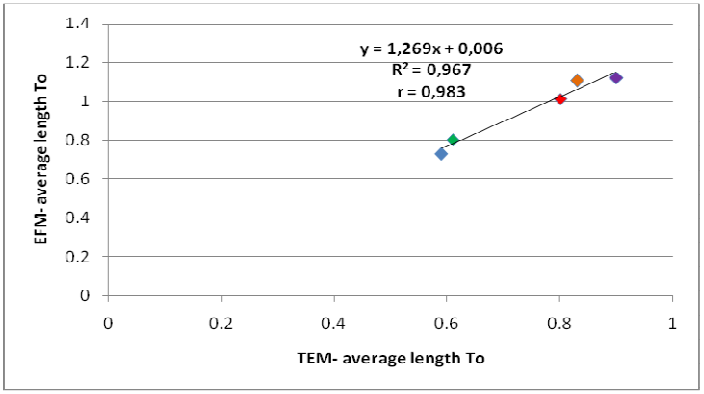

Figure 1. Correlation between TEM and EFM values of the average length cells at the time zero, in the five microcosms. Continuous line represents the linear regression: y=1.269x + 0,006 (r = 0.983; n = 5)

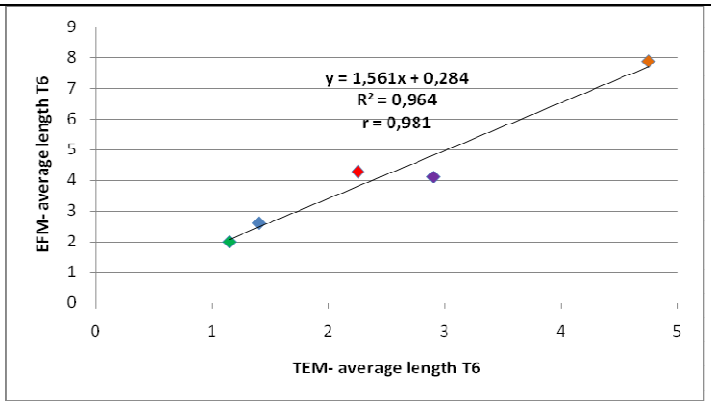

Figure 2. Correlation between TEM and EFM values of the average length cells at 24 hours of incubation time in the presence of nalidixic acid, in the five microcosms. Continuous line represents the linear regression: y=1.561x + 0,284 (r = 0.981; n = 5)

The results show a good linear correlation between cell length measurements obtained by both methods (TEM and EFM), with the specification that the size cells based on EFM method is generally higher than that obtained by TEM. This could be attributed to use of different magnification of the images in both methods, and different methods to prepare the biological probes for either EFM or TEM (Chen et al., 2001). The dehydration step could produce a decrease in cell dimensions (Wang **et al., 2000; Wrigley N.G., 2004).**

Our results showed a variation in time of the size of individual bacterial populations in the five natural microcosms.

These investigations allowed us to highlight the very small coccoid forms (diameter < 0.25 µm) when they were viewed by epifluorescence; however, when inspected by TEM one can see that some of the cells are actually rods and vibrio (Figure 3).

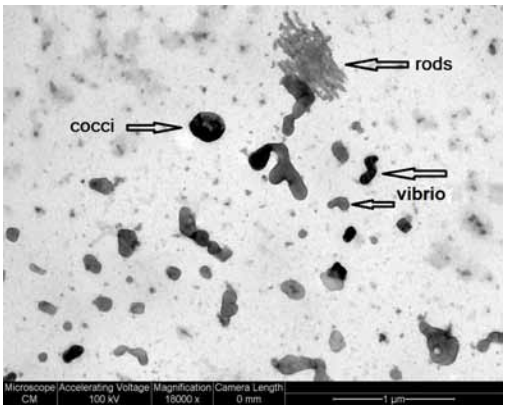

Figure 3. Minibacteria: rods, cocci and vibrio (diameter < 0.25 µm)

The length of these rods and vibrio obtained by transmission electron microscopy measurements is likely to exceed 0.5 µm.

In Figure 4 are the few examples of measurements performed by TEM on bacteria within the microcosms. In microcosm 2 at zero time average cell length was 0.800 µm, but after 24 hours incubation, the average cell length was 2.250 µm; however in M4 where there was an additional nitrogen source, the average cell length was 4.750 µm at T_6.

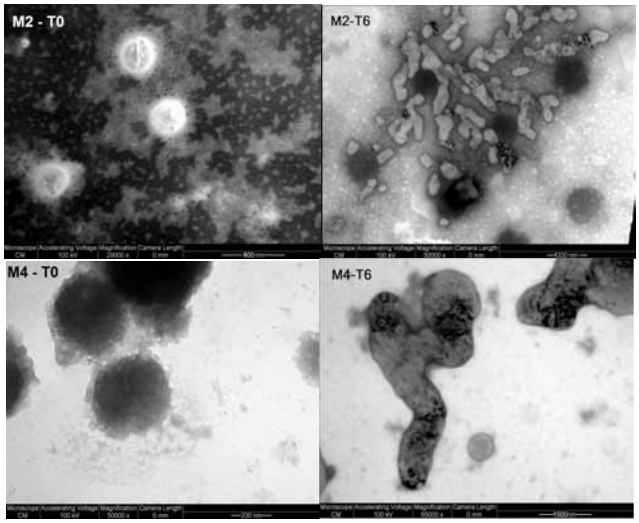

Figure 4. Bacterial cell size in M2 and M4 measured by TEM at T_0 (0 hours) and T_6 (24 hours).

The experimental TEM results illustrate the presence of granules (Figure 5) in analyzed cells.

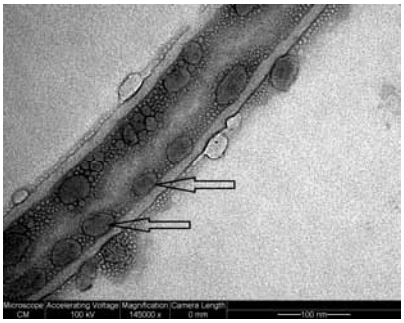

Figure 5. Section in the cell wall (x 145.000). The arrows indicate small grains observed at the surface and inside the cell.

It is difficult to interpret the electron diffraction for a particular crystalline phase. In M4 sample there is the possibility that the grains could be polyhydroxybutyrate (PHB) because of the presence of the crystalline phase with orthorhombic structure, with network

parameters 5.77 Å, 13.2 Å and 5.97Å. The SAED images of these unidentified components show presence of mixed phases, amorphous and crystalline, with some lines that can be indexing with PHB orthorhombic structure (microcosm 4 at T_0 lines (021), (040), (131), (211), (240), (233), (304) and microcosm 4 at T_6 (120), (121), (040), (022), (122), (132), (042), (241), (013), (033), (331), (203), (014), (442), (423), (334)) with both sample exhibit a maxima at (040) line. The average crystalline size obtained using Debye-Scherrer relation is 4.6 nm for sample 4 T0 and 5.4 nm sample 4 T6 (Oprea et al., 2008; Prodan & Ciupină, 2010).

Further results obtained by EFM using the specific staining for PHB using Nile blue (Ostle & Holt, 1982) further confirm that the grains, the inclusions contain PHB (figure 6).

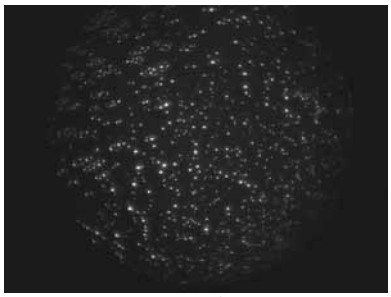

Figure 6. Nile blue staining of PHB observed by EFM

Thus, the presence of PHB is argued both by TEM and EFM (Mercan, 2002; Betscheider & Jose, 2009), the obtain results suggesting that under these conditions the cells (that are not able to divide because of the presence of nalidixic acid) are in position to synthesize PHB, probably as an expression of an imbalance between nitrogen and organic carbon.

4. CONCLUSIONS

1. There is a good linear correlation between cell length obtained by measuring the cell using two different methods, namely epifluorescence microscopy and transmission electron microscopy (r = 0.983).
2. The presence of PHB in these cells is argued both by TEM and EFM, suggesting an excess of organic carbon source as compared with nitrogen source.

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THERAPEUTIC EFFECT OF MONASCUS METABOLITES

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Abstract. *Many reports indicate the therapeutic effects for red yeast rice [1-3]. From this point of view, experiments performed on cell line type Hep2 and WiDr indicated an inhibitory effect on cancer cells. Another study performed on murin cell type RAW 264.7 stimulated with LPS reveal the inhibition of nitrogen oxide production in comparison with quercetin used as witness. These properties is due to presence of some compounds like monakarin A-F, monacolin K, dimerumic acid, monascopyridine C-D, xanthomonasin A-B, monascumic acid, which exists in metabolites produced of Monascus and the presence of ascorbic acid and polifenols. Our study performed with alcoholic red yeast rice extracts in the presence of 1% of collagen, reveals a powerful antioxidant effect, in which the quenching ratio is 95% in comparison with luminol, used as witness. Studies performed in vivo, regarding cicatrisation effect on mouse with the same sample (alcoholic extract of red yeast rice and 1 % collagen) indicate a potential cicatrisation effect, probably due to presence of glucosamine compound which acts as cicatrisation factor.*

Keywords: Monascus metabolite, therapeutic effect

1. INTRODUCTION

The pigments from Monascus metabolites, named red Monascus or Monascus, represent a mixture of lypophylic azaphylone. These mixtures contain yellow pigments (ankaflavin and monascin), their oxidised compounds respectively orange pigments (monascorubrine and rubropunctatine) and corresponding compounds with N (red pigment) rubropunctamine and monascorubramine, which derived from isochromene (figure 1) [1-4]. The polyketidic chains of azaphylones was asambled from acetic acid (which represent the starter compound) and 5 molecules of malonic acid (units of chain extension) [5-8].
The chromophores from hexaketidic compounds were obtained via monascusone A. The external chains of this compounds constitute a closed system of gama lactons obtained with condensation of 2-oxohexanoic acid with 2 –octooctanoic acid derived from fatty acids. The Nitrogen analogues of azaphylones were obtained by their reaction with aminoacids.
Another type of compounds produced of *Monascus sp*. (named monascotinates) was identified from ethyl acetate extracts of Monascus pilosus BCRC 38093 metabolites. These compounds have the structure presented in figure 2 and reveals the following spectral characteristics:

Monasnicotinate A (1). Yellow oil. UV $_{max}$ (MeOH): 253, 280, 330 nm. IR $_{max}$ (near): 1668, 1712 cm^{-1} (C=0). ESI-MS: 380 ($[M+Na]^4$). HR-ESI-MS: 380,1837 ($[M+Na]^+$, $C_{2i}H_{27}NaN0_4^+$; calc. 380,18353).

Monasnicotinate B (2). Yellow oil. UV $_{max}$ (MeOH): 248, 275, 338. IR $_{max}$ (near): 1676, 1716 cm^{-1} (C=0). ESI-MS: 394 ($[M+Na]^4$). HR-ESI-MS: 394,1994 ($[M+Na]^+$, $C_{22}H_{29}NaNO_4^+$; calc. 394,1998).

Monasnicotinate C (3). Yellow oil. UV $_{max}$ (MeOH): 251, 282, 327 nm. IR $_{max}$ (near): 1672, 1724 cm^{-1} (C=0). ESI-MS: 408 ($[M+Na]^4$). HR-ESI-MS: 408,2151 ($[M+Na]^+$, $C_{22}H_{29}NaNO_4^+$; calc. 408,2153).

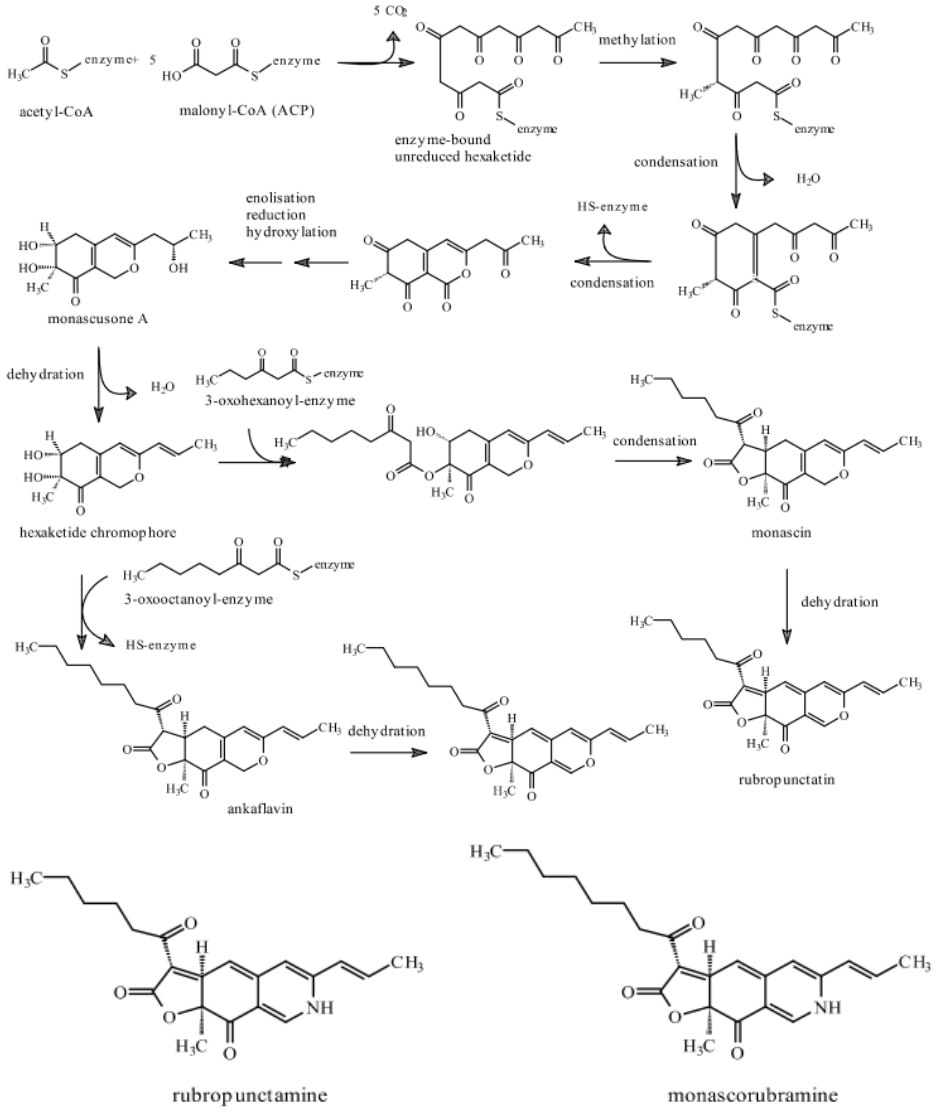

Figure 1. Monascus azaphylone formation

Monasnicotinate D (4). Yellow oil. UV $_{max}$ (MeOH): 245, 271, 328. IR $_{max}$ (near): 1665, 1716 cm^{-1} (C=0) ESI-MS: 382 ($[M+Na]^4$). HR-ESI-MS: 382,1994 ($[M+Na]^+$, $C_{22}H_{29}NaNO_4^+$; calc. 382,1994).

1 R_1 = CH=C($COCH_3$)$CH_2CO(CH_2)_4CH_3$, R_2 = $COOCH_3$, R_3 = (E)CH=$CHCH_3$

2 R_1 = CH=C($COCH_3$)$CH_2CO(CH_2)_4CH_3$, R_2 = $COOC_2H_5$, R_3 = (E)CH=$CHCH_3$

3 R_1 = CH=C($COCH_3$)$CH_2CO(CH_2)_6CH_3$, R_2 = $COOCH_3$, R_3 = (E)CH=$CHCH_3$

4 R_1 = CH=C($COCH_3$)$CH_2CO(CH_2)_4CH_3$, R_2 = $COOCH_3$, R_3 = *n*-C_3H_7

Fig. 2 General formula of monasnicotinates [7]

Another researchers identified in acetone extract which contains orange azaphylones, another 4 compounds, presented in figure 3, named monaphilol A-D, compounds which give the florescence.

Biological evaluation indicated that compounds 1–4 inhibited nitric oxide (NO) production on lipopolysaccharide-stimulated RAW 264.7 cells. Compounds 1–4 also exhibited antiproliferative activities against human laryngeal carcinoma (HEp-2) and human colon adenocarinoma (WiDr).Main characteristics of monaphilols is presented in the following section.

Monaphilol A (1): orange dark oil; [ ] $_D^{25}$ = 2648,8° (c= 1,23, acetone); UV (MeOH) $_{max}$ (log *):* 469 (4,0), 302 (3,4); IR $_{max}$ (KBr) 3401, 2954, 2918, 2859, 1742, 1655, 1528, 1437, 1239, 1168, 1073, 902, 827 cm^{-1}; ESIMS *m/z*385 $[M + H]^+$; HRESIMS *m/z*385.2016 $[M + H]^+$ (calc. 385.2015; $C_{23}H_{29}O_s$).

2. MATERIAL AND METHODS

The aims of the paper is to evaluate the potential therapeutic effect of e Monascus sp.metabolited (by antioxidant and cicatrisation study).Monascus metabolite extracte was obtained by dissloved the red powder (obtained in solid state biosynthesis betwen Monascus sp. and on rice) in ethanol.

Materials. The luminol (LH_2) - hydrogen peroxide (H_2O_2) system with concentration of LH_2 = 2.5 x 10^{-5} M and H_2O_2 = 30 mM in Tris-HCl buffer, pH 8.10, was considered as reference system. The Monascus metabolites (10% collagen in water and 50% Monascus metabolites) was diluted in water and ethanol of spectroscopic grade; The stock solution was: 200 l pigment at 3 ml of water and ethanol, respectively.

Methods and apparatus. Absorption spectra have been performed with a Perkin Elmer Lambda 35 Spectrometer. The chemiluminescence measurements have been

performed with a TD 20/20 chemiluminometer Turner Design. Measurements were carried out in five replicas and averaged, obtaining a relative scattering of the results of up to 10% of the average value; The working volume was 1000 µl.The antioxidant activity of the pigment, A %, was calculated according to the equation:

$$A\ (\%) = \frac{(I_0 - I)}{I_0} \times 100 \quad (1)$$

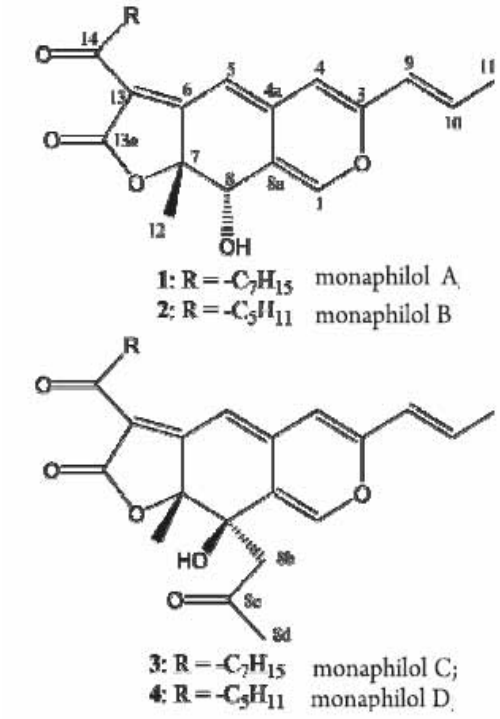

Fig.3
Structure of monaphiloles A-D [8].

Monaphilol B (2): orange dark oil [ ] $_D^{25}$=2581,8° (c=0,33, acetona); UV (MeOH $_{max}$ (log *):* 469 (4.0), 303 (3,4); IR $_{max}$ (KBr) 3398, 2958, 2930, 2851, 1734, 1655, 1524, 1441, 1263, 1239, 1172, 1073, 906, 796 cm^{-1}. ESIMS *m/z* 357 [M + H]$^+$; HRESIMS *m/z* 357.1698 [M + H]$^+$ (calc. 357.1702; $C_{21}H_{2S}O_s$).

Monaphilol C (3): orange dark oil; [ ] $_D^{25}$ = 2095,7° (c= 3,49, acetona); UV (MeOH) $_{max}$ (log *):* 470 (3.9), 307 (3.4); IR $_{max}$ (KBr) 3366, 2958, 2926, 2855, 1746, 1663, 1528, 1449, 1358, 1239, 1073, 958, 835 cm^{-1}; ESIMS m/z441 [M + H]$^+$; HRESIMS m/z441.22S6 [M + H]$^+$ (calc. 441,2277; $C_{26}H_{33}O_6$).

Monaphilol D (4): orange dark oil [ ] $_D^{25}$ = 1491,1° (c= 0,45, acetona); UV (MeOH, $_{max}$ (log :*)* 470 (4,0), 307 (3,4); IR $_{max}$ (KBr) 3366, 2958, 2926, 2859, 1738, 1667, 1528, 1441, 1362, 1235, 1168, 1069, 891,807 cm^{-1}; ESIMS *m/z* 413 [M + H]$^+$; HREIMS *m/z* 413.1997 [M + H]$^+$ (calc. 413,1964; $C_{24}O_{29}H_6$).

Where I_0 and I represent CL intensity measured for the reference system and for the reference system in the presence of the pigment, respectively. Both values were measured 5s after the beginning of the reaction. The fluorescence emission spectra of Monascus metabolites were recorded with Jasco FP-6500 Spectrofluorometer.

Cicatrisation effect was investigated in vivo,on Wistar mice using a Monascus extract conditioned with IPM (isopropyl myristate)

3. RESULTS AND DISCUSSION

Optical spectroscopic characteristics of the pigment were investigated. The solubility of the pigment is better in ethanol than aqueous solution. For the pigment in ethanol, it can be seen in Figure 4, two absorption bands at 374 and 508 nm respectively. Using different excitation wavelengths, the fluorescence emission of the pigment was evidenced. Figure 5 shows the fluorescence emission spectra of the Monascus metabolites in water (Figure 5A) and ethanol solution (Figure 5B). Three well structured emission bands were evidenced at 430, 455 and 525 nm respectively in aqueous as well as in ethanol solution. Figure 6 presents the fluorescence emission spectra of the Monascus metabolites in the chemiluminescent system luminol- hydrogen peroxide, in Tris-HCl pH 8.10, in direct comparison with the reference system and also with the Monascus metabolites in ethanol solution. For the LH_2/Tris-HCl, pH 8.10/ H_2O_2, the fluorescence emission at 424 nm corresponds to LH_2 fluorescence emission. The red shifted emission of 10 nm (434 nm) in the presence of the pigment was evidenced. In direct comparison with the free Monascus metabolites in ethanol solution, it was observed that the fluorescence emission band at 525 nm decreases. The feature may be due to the oxidation of the metabolites by hydrogen peroxide.

By the chemiluminescence method, the antioxidant activity of the pigment was estimated, (results presented in Table 1). The antioxidant activity found around 90% is attributed to a good stability to the oxidative processes. This behavior is also correlated with the increasing in the collagen concentration.

Table 1. The chemiluminescence (CL) parameters in the LH_2 - H_2O_2 system, in Tris-HCl buffer, pH 8.10 (Reference system) (RS) in the presence of the pigment.

System	**I_{CL}, a.u.**	**A, %**
RS	1799.05	-
2% Monascus metabolites	496.9	72.38
4% Monascus metabolites	252.05	85.99
6% Monascus metabolites	185.05	89.71
8% Monascus metabolites	96.55	94.63
15% Monascus metabolites	75.095	95.82

Tests regarding cicatrisation effect. In the first step, the witness of conditioned reagent (respectively isopropyl myristate and water) was tested in order to establish his influence regarding scars retraction. Result obtaining (data not shown) reveals no influence of conditioning reagent. Regarding bioproduct derived from solid state (P), the average value of surface lesion with the first day was 3,8 cm^2, in the 3-days 2,84 cm^2 and in the days 14 and 18, the average value was more accentuated for bioproduct which contain Monascus sp.metabolite (P) in comparison with conditioning reagent (figure 7). These results indicate a possible cicatrisation effect of Monascus sp. metabolite obtained in solid state biosynthesis.

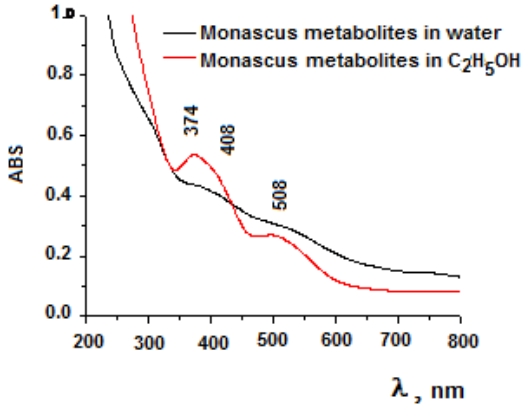

Figure 4. The UV-Vis Absorption spectra of the pigment in water and ethanol solutions.

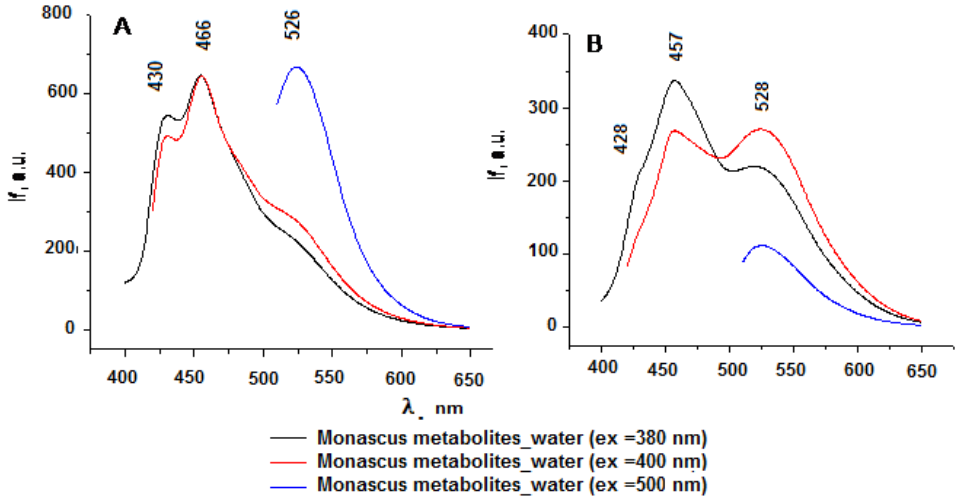

Figure 5. Fluorescence emission spectra of the Monascus metabolites in water (A) and in ethanol (B) solutions at different excitation wavelengths.

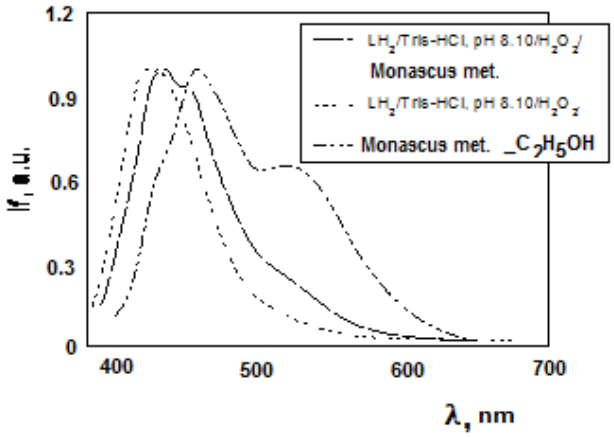

Figure 6. Normalized Fluorescence emission spectra of the pigment in the luminol (LH_2) (2.5×10^{-5}M) – hydrogen peroxide (30 mM), in Tris-HCl buffer (pH 8.10).

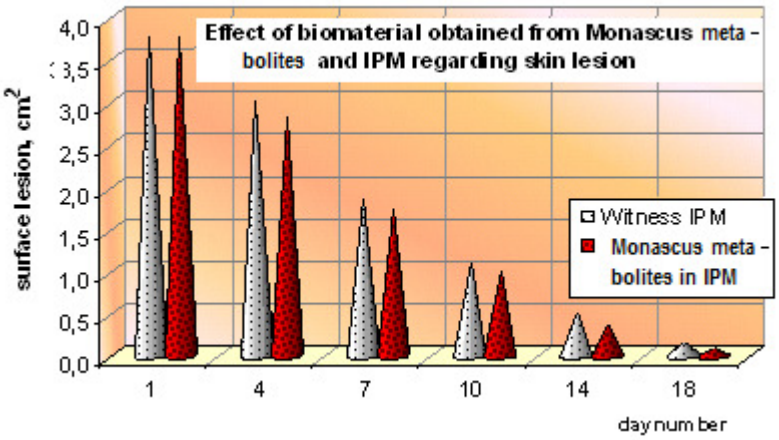

Figure 3 Cicatrisation effect of biomaterial obtained from Monascus

4. CONCLUSION

Optical spectroscopic characteristics of the pigment reveal the following: by UV-VIS investigation, in etanol media, two absorption bands at 374 and 508 nm was obtained respectively. Using different excitation wavelengths, in fluorescence emission three well structured emission bands were evidenced at 430, 455 and 525 nm respectively in aqueous as well as in ethanol solution.

Antioxidant activity of the pigment was found around 90% is attributed a good stability to the oxidative processes. The results reveal the positive effect recordered by M*onascus*

sp.metabolites. In this case, this product increased the rate of cicatrisation process with 70% in comparison with placebo tests in the case in which these bioproducts was administered in the 4 day of post skin excision. The improvement of scars retraction can be due to presence of some compound based on glucosamine derivate present in the Monascus metabolites. Further study are needed to determine the therapeutic effect of other active component from red yeast rice, including sterols, isoflavones and tannins.

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SECTION V: FOOD SAFETY

NEW PACKAGING MATERIALS AND THEIR EFFECT ON THE SHELF-LIFE OF PACKAGED SALAD

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Abstract. *The quality of fresh products, minimally processed, such as salad, depends on preparation method, packaging material and storage conditions. Packaging material affect sensory properties of salad, because the intensity of respiration and color change of the product depend on the composition of the used material.*
Depending on the product, process and storage conditions the microbiological shelf-life may be determined by either the growth of spoilage or pathogenic microorganisms. Traditional methods for the determination of shelf-life include storage of the product at different temperatures and determining spoilage by sensory evaluation or microbial count. This will involve the natural flora of the product, which may vary between batches. For products where the shelf-life may be set by the growth of pathogenic microorganisms this may involve challenge testing the product with the organism prior to storage and microbial analysis at intervals.
In the experiments was used salad – chopped fresh leaves, packed in normal atmosphere, using different types of new packaging materials: nanocomposite-based LDPE/modified silicate monolayer films containing starch and hydrolyzed collagen and usually PP films. Bags containing salad were stored at 5 Celsius degrees.
Packaging material effect on product was followed by microbiological and sensory analysis during storage up to 10 days under mentioned conditions. Sensory evaluation parameters included: color, browning, odor, degradation and overall quality. Microbiological and sensory analyses results were correlated with the permeability properties to O_2, CO_2 and water vapor.
The best option may be considered LDPE/silica-Amp for which can be estimate a shelf-life longer than 7 days obtained with PP films currently used.

Keywords: shelf-life, minimally processed food, packaging

1. INTRODUCTION

Lettuce is an important leafy vegetable and one of the main sources of dietary carotenoids (Liu & al. [1], Moeller & al. [2]). The cut and packaged lettuce for food service and salad mixes are some increasingly important components of the produce industry. Increased demand for the convenient, pre-cut salads and lettuce has led the scientists to search for ways to select lettuce cultivars that stay fresh, colorful, and crisp. Shelf life and visual quality of fresh-cut lettuce are affected by many things, including production environment, vegetative maturity, and type of lettuce chosen. Although an increasing

variety of lettuce types are being grown, romaine and "crisp-head" (such as iceberg) are the most widely produced for salad-cut products.

One of the major problems facing food manufacturers is responding rapidly to the demands of the major retailers who supply a large proportion of the market.

The salad leaves, as other leafy vegetables, damage themselves quite rapidly during the storage period, because of their increased respiration rate (Soliva & Martin [3]). The low shelf life of fresh-cut minimally processed lettuce leaves is one of the major problems that the producers are facing with (Brecht & al. [4], Saltveit [5]). As so, the quality of made up products depends on the method of their preparation, packing material and storage conditions (Barry-Ryan [6]). Using efficient packaging materials is now necessary for all types of fresh or processed foods. All the qualities and attributes of a product, that have been developed during production processes can be lost if it the product doesn't reach the consumer in good conditions.

Packing materials influence sensory properties of products, because lettuce respiration intensity and product color changes depend on the composition of the used materials (Del Nobile & al. [7]).

One of the major problems facing food manufacturers is responding rapidly to the demands of the major retailers who supply a large proportion of the market.

MAP can be defined as an active packaging method that creates a new atmosphere inside the package, resulting in a delay of chemical damage and at the same time, inhibit growth of microorganisms perish. The main gases used are oxygen, carbon dioxide and nitrogen. Carbon monoxide has limited applications in MAP, but sulfur dioxide is used more frequently.

2. MATERIALS AND METHODS

Sample preparation

Two types of lettuce were analyzed: Type A – imported "Endivia mix/Latino mix" (kindly provided by Eisberg Romania), known to be quite sensible at O_2 concentrations and type B – lettuce from a local supplier. The samples were represented by lettuce leaves. After they were washed with cold tap water, the excess of water was manually removed.

Samples containing 180 grams each from type A lettuce were packaged in nanocomposite-based LDPE/modified silicate monolayer films and samples containing 50 grams each from type B lettuce were packaged in BOPP films (with and without perforations).

There were used bags with dimensions of about 30x20 cm. The bags were hermetically sealed and stored at 4-5°C. The samples were observed for 10 days.

Films characterization

The following films: nanocomposite-based LDPE/modified silicate monolayer films containing starch (D20A-Amp) and hydrolyzed collagen (D20A-C08G), LDPE film, and usually BOPP films (with 5 mm perforations and without perforations) were tested in terms of barrier properties. The barrier properties to water vapor and gases expressed by the permeability were determined by specific analysis: determining transmission rates of water vapor according to DIN EN ISO 15106-1: 2005 (method of moisture detection) and determining gas transmission rate through plastic films and foils according to DIN 53380-1

(manometric method). These tests were made using Lyssy L80-5000 water vapor permeability tester and Lyssy L100-5000 manometric gas permeability tester both from PBI Dansensor.

Physico-chemical analyses

Water activity was determined at baseline, before the washing process, and that on every testing day, using the Aquaspector AQS-2-TC device.

Homogeneous samples were obtained by cutting the lettuce leafs with a sterile scalpel.

Evidence of weight loss as a result of dehydration vegetables during storage was determined by weighing with the electronic balance Kern EW1500-2M. Salads were first weighed.

To determine the pH 10g of homogeneous sample were weighed, then 100mL distillated water were added. It was used the Inolab pH meter with combined electrode and the samples were homogenized using a Circular Seward 400 Stomacher.

Microbiological analyses

Samples were initially analyzed, after the washing process, considered day 0, and then on day 1, 3, 5, 7 and 10. For the microbiological analyses, 10g of lettuce were weighed and then homogenized with 90mL peptone water using the Circular Seward 400 Stomacher for 30 seconds. Serial dilutions (1:10) of each homogenized sample were made. Total aerobic mesophiles and psychrotrophs were determined according to SR EN ISO 4833/2003, using Plate Count Agar (PCA, Biokar Diagnostics) and incubated at 30°C for 3 days. The yeasts and moulds were determined according to SR ISO 21527-1/2009, using Dichloran-Rose Bengal Chlormaphenicol agar (DRBC agar, Biokar Diagnostics), incubated at 25 °C for 5 days. Coliform bacteria were determined using Violet Red Bile Agar with Lactose, VRBL (Biokar Diagnostics) – incubated 24h at 37 °C STAS ISO 4832/2009. *Escherichia coli* was determine according to SR ISO 16649-2/2007 using Tryptone Bile Glucuronide, TBX (Lab M Harlequin) – incubated 24h at 44 °C.

Sensory quality

There were evaluated changes in visual quality, texture, flavour, off-odours and browning.

3. RESULTS AND DISCUSSIONS

Barrier properties analysis

The barrier properties to water vapor and gas (oxygen, nitrogen and carbon dioxide) are presented in *Table 1*.

Effect of storage on water loss

In terms of water activity, there was neither significant difference between the salads packaged in different film bags, and the perforations didn't have any significant relevance. The initial lettuce water activity, at baseline, before washing, was 1.023. The values registered on the testing days are listed in table 1.

The lowest water activity values were registered for sample 1, especially in the first days of storage. In fig. 1 there can be observed that all the tested lettuce samples registered an increase of the water activity values in the first 3 days, reaching the maximum

point on the fourth day of storage, and then, a decrease in values was observed for both samples. It was observed that all tested samples had the same evolution regarding the differences for the water activity values during storage period.

Table 1 Permeability of tested films

Sample	Thickness (mm)	Permeability				
		Water vapor permeability ($g/m^2 \cdot day \cdot atm$)		Gas permeability ($cm^3/m^2 \cdot day \cdot atm$), 23 ºC, 0%RH		
		23 ºC, 15% RH	38 ºC, 10% RH	O_2	N_2	CO_2
LDPE	0,040	5,02	9,46	4484	973,83	10916
D20A-C08G	0,050	4,16	7,29	1720	506,39	5870
D20A-Amp	0,040	1,72	5,74	1352	466,02	5261
BOPP/BOPP	0,050	2,55	9,09	1231	267,61	3324

Table 2 Water activity values for type B packaged lettuce

Sample	Water activity values					
	Day 1	Day 2	Day 4	Day 5	Day 7	Day 10
BOPP	1.036	1.061	1.099	1.114	1.101	1.072
BOPP perforated	1.049	1.080	1.107	1.118	1.091	1.069

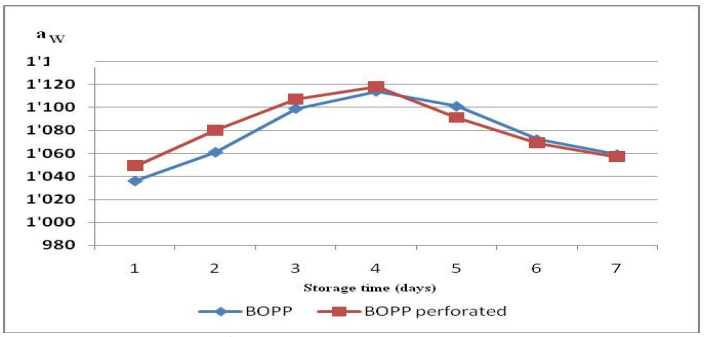

Figure 1 Evolution of water activity values during the storage period

Microbiological stability

In both cases, the microbial parameter *Escherichia coli* was not found, and the results were expressed as <10 cfu/g.

Figures below show the viable cell concentration of total mesophilic bacteria (*Figure 2*), yeasts and moulds variation (*Figure 4 and 5*) and coliforms bacteria variation

(*Figure 6 and 7*), during the observed period for both "Endivia mix"lettuce (type A) and green lettuce type B).

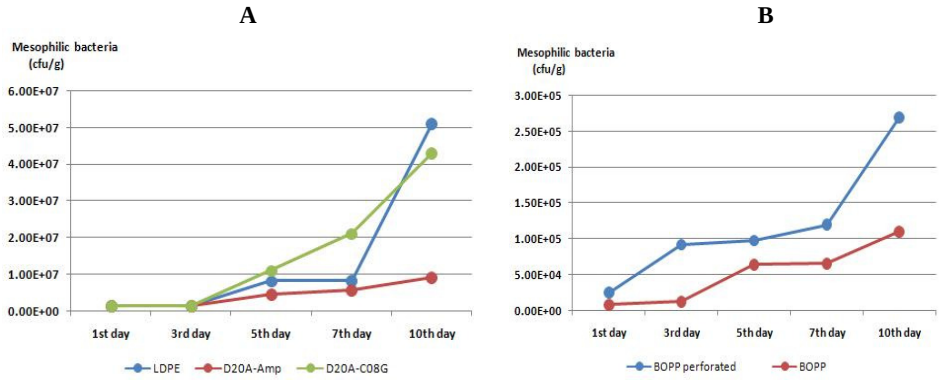

Figure 2 Mesophilic bacteria cell concentration during storage time for both types of lettuces (Endivia mix – A and green lettuce – B)

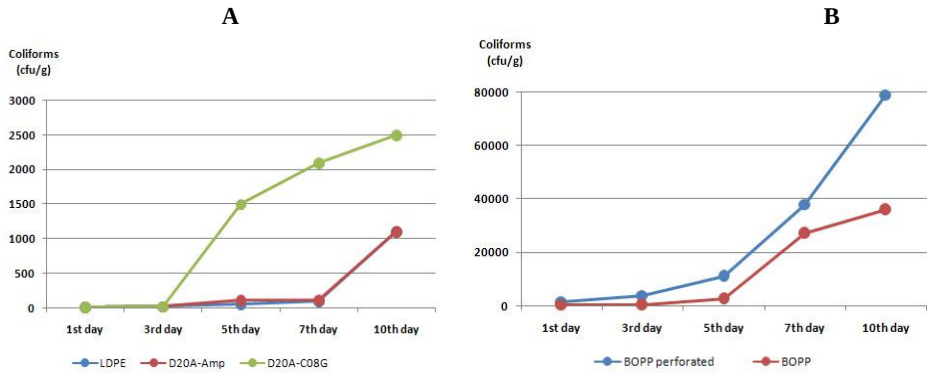

Figure 3 Coliforms bacteria variation during storage time for both types of lettuces (Endivia mix – A and green lettuce – B)

A **B**

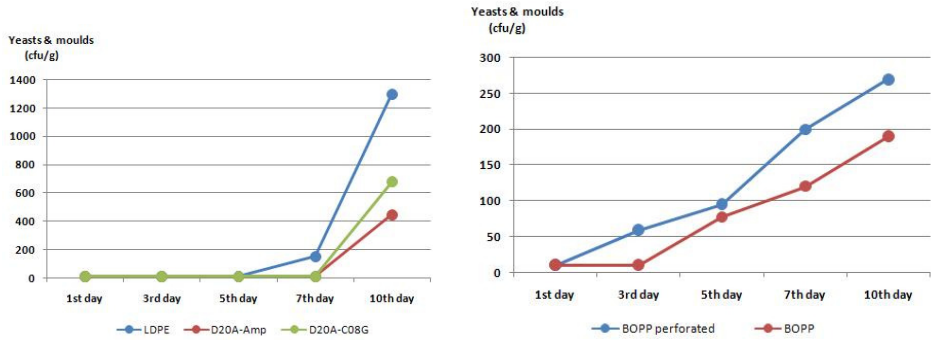

Figure 4 Yeasts and moulds variation during storage time for both types of lettuces (Endivia mix – A and green lettuce – B)

During storage, there were high values for mesophilic bacteria cell concentration for type A lettuce, as an evidence of increased sensitivity of salad mix compared with green lettuce, related to minimal processing (cutting, washing).

Regarding BOPP film, using a perforated film (Ø 5mm), did not lead to favorable results in terms of microbiological results.

There was a microbiological degradation of green lettuce starting the day 4 of storage (higher values for yeasts and molds, total coliforms bacteria and mesophilic bacteria). The microorganisms growth was according with increased water activity, starting with day 4 of storage. It must be considered the lower value of water vapor permeability of BOPP film. Regarding Envidia mix lettuce, number of the coliforms bacteria, an indicator for safety of the product, is not considered representative for the comparative study of the three materials. But after 7 ddays of storage, the lettuce packaged in LDPE film was maintained at the lowest value. We mention that the yeasts and molds value remained lower than 10 cfu/g for 7 days, for both nanocomposite-based LDPE films and increased only for LDPE film.

In all cases no molds contamination was observed, but only with yeast.

Sensory properties

Initial properties of the lettuce:

- Appearance: without signs of infection or foreign bodies;
- Color: yellow-green to dark green;
- Taste and smell: pleasant, slightly bitter, fresh lettuce, without strange taste or smell.

Sensory modifications noted during the storage period are presented in *Table 3 and 4*.

Table 3 Sensory modifications of Endivia mix lettuce

Date	Sample	Appearance	Color	Taste and smell	Observations
Day 1	LDPE	No signs of infestation	Specific - From yellow-green to dark green	Pleasant, slightly bitter, specific fresh lettuce	
	D20A- Amp				
	D20A- C08G				
Day 3	LDPE	No signs of infestation	Specific - From yellow-green to dark green	Pleasant, slightly bitter, specific	
	D20A- Amp				

	D20A- C08G			fresh lettuce	
Day 5	LDPE	No signs of infestation	Specific - From yellow-green to dark green	Pleasant, slightly bitter, specific fresh lettuce	
	D20A- Amp				
	D20A- C08G				
Day 7	LDPE	No signs of infestation	Specific - From yellow-green to dark green	Pleasant, slightly bitter, specific fresh lettuce	
	D20A- Amp				
	D20A- C08G				
Day 10	LDPE	Damaged leafs - 10%;	Brown leaves 2%	Unmodified	Condensation on the bag surface - 5%
	D20A- Amp	Damaged leafs - 1%;	Specific - From yellow-green to dark green	Unmodified	
	D20A- C08G	Damaged leafs - 2%;	Specific - From yellow-green to dark green	Unmodified	Condensation on the bag surface - 20%

Analyzing *Table 3* results:

- After 7 days of storage, no differences were observed between the three films (LDPE, D20A-Amp and D20A-C08G);

- Of the 10th day differences were found between D20A-Amp and D20A-C08G film and, compared to LDPE, is reported the browning (2%) and degradation (10%) of lettuce leaves (cca.10%), more powerful in LDPE film, not yet observable in the other two films.

- From the 10th day condensation was also observed, to a greater extent in LDPE (bag surface cca.20%), to a lesser extent in D20A-C08G film, respectively unobservable in D20A-Amp film.

Sensory analysis results are correlated with oxygen, carbon dioxide and water vapor permeability properties.

Lettuces packaged in D20A-Amp and D20A-C08G films reached a suitable level of O_2 and CO_2 inside the bag, so the lettuces stored in these bags maintaining their freshness for the entire storage period (10 days).

In *Figures 5* and *6* are presented the three samples of Endivia lettuce (type A) after 7 and 10 days of storage.

Table 4 Sensory modifications of green lettuce

Date	Sample	Appearance	Color	Taste and smell	Observations
Day 1	BOPP	No signs of infestation	Specific – light-green	Pleasant, slightly bitter, specific fresh lettuce	
	BOPP perforated				
Day 3	BOPP	No signs of infestation	Specific – light-green	Pleasant, slightly bitter, specific fresh lettuce	
	BOPP perforated				
Day 5	BOPP	No signs of infestation	Specific – light-green	Pleasant, slightly bitter, specific fresh lettuce	
	BOPP perforated				

Day 7	BOPP	Damaged leafs - 10%;	Specific – light-green	Pleasant, slightly bitter, specific fresh lettuce	Condensation on the bag surface - 10%
	BOPP perforated	Damaged leafs - 10%;	Brown leaves 20%	Unmodified	
Day 10	BOPP	Damaged leafs - 30%;	Brown leaves 10%	Pleasant, slightly bitter, specific fresh lettuce	Condensation on the bag surface - 20%
	BOPP perforated	Damaged leafs - 50%;	Brown leaves 35%	Unmodified	

Figure 5 Packaged Endivia mix lettuce after 7days of storage

Figure 6 Packaged Endivia mix lettuce after 10 days of storage

In *Figure 7* are presented the two samples of green lettuce (type B) after 3 days of storage. As described in *Table 4* from this figure we can see no modifications regarding sensory attributes (appearance and color).

Figure 7 Packaged green lettuce after 3 days of storage

4. CONCLUSIONS

During storage, for the lettuce packaged in BOPP film, the water activity increased until day 4; this can be correlated with lower values recorded for water vapor permeability.

Sensory analysis results are correlated with oxygen, carbon dioxide and water vapor permeability properties.

Lettuces packaged in D20A-Amp and D20A-C08G films reached a suitable level of O_2 and CO_2 inside the bag, so the lettuces stored in these bags maintaining their freshness for the entire storage period (10 days).

During storage, for Envinia mix lettuce (type A), higher values for total mesophilic bacteria were recorded, as evidence of increased sensitivity of Endivia lettuce compared with green lettuce, related to minimal processing (cutting, washing).

The results indicated that the permeability of the packaging film affects the atmosphere inside the bag, product quality and shelf life.

After completing the experiments, comparative analysis of the two types of lettuce salmples showed a higher microbiological stability for type A (Endivia mix lettuce), up to 10 days of storage; D20A-Amp film is considered a viable alternative for lettuce packaging.

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THE EFFECTS OF THE COMPONENTS SPECIFIC MIGRATION FOR THE FOOD STUFF CONSERVATION IN METALLIC CANS

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Abstract. *Being known the effects of the components migration from the package in the food product it is necessary to be determined the degree of stability of the protection lacquers that are found in the structure of food metallic packagings.*
The goals of the paper were:
- the detection and quantification of certain migrants with toxic potential from certain inner side protection lacquers of the food metallic cans;
- the analysis of the temporal stability of the lacquer layer in the case of the metallic cans for certain food products.
The approaching methods comprised not only the analysis of the package but also that of the product preserved. The specific components migration (bisphenol A) in food simulants was analyzed in two ways: the UV-VIS method and the GC-MS method and the analysis of the package heavy metal migration as well as the migration of the heavy metals from the product by the atomic absorption spectroscopy (AAS).
From the analysis of the inner side of the can and the organoleptic and physico-chemical analysis of the product „Fois gras" the yellow lacquer type we observed that the phenomena is more accentuated than in the case of the product „Pork meat in natural juice". This thing wasn't seen in the cases with white lacquer.

Keywords: ***metallic cans, tin layer, white lacquer, yellow lacquer, AAS, GC-MS, UV-VIS***

1. INTRODUCTION

The anticorrosive protection materials used in the food domain ensure the safety of the metal surfaces in front of the atmospheric corrosion, the protection from the reaction with the food product contained and the protection of the food contained. The protection of the food stuff contained from metal contamination contributes at the reduction of the costs along the time and at the protection of the environment. [6,7]

The safety of the packaging materials is based on the fact that during the food stuff contact the unsure chemical substances don't migrate from the material to the food stuff.

From all the material types the epoxyphenolic lacquers are the most important being universally used for body cans as well as for sealings, for two or three pieces metallic cans as well as for pressed cans. [1]

The epoxyphenolic lacquers based on bisphenol A (BPA) are often called GOLD phenolic lacquers due to their yellow-glodish specific colour and are used for most of the applications that include extremely corrosive food stuff. [2]

The establishing of the sterilization methods as well as their correct application are very important for the slightest mistakes regarding the sterilization formula (not respecting the time, temperature and pressure conditions) can have severe consequences upon the preservation and the quality of the product. At high temperature (sterilization) the resins that compose the protection lacquers can decompose and result in components with high toxic potential (bisphenol) and can migrate from the package to the food stuff. [3,4]

The traditional method for fabricating food cans in the food can industry implies the using of tin covered with protection lacquers. This trend is in a continuous change due to the using reglementations (european and national) regarding the dangerous endocrine effects of the bisphenol found in the protection lacquers (Moller şi alţii, 2004). The frame reglemenation *1935/2004/CE*, as well as the new CE directives regarding the materials that come in contact with the food stuff impose migration limits for the specific components. On national level we can mention HG nr. 1197/2002 for the approving of the Regulations regarding the materials and objects that come in contact with the food stuff and the additional things regarding the using of the epoxy derivates in materials and objects that come in contact with the food stuff. [5] Being known the effects of the components migration from the package in the food stuff it's necessary to determine the degree of stability of the materials by global migration and specific migration testes for the components.

2. MATERIALS AND METHODS

We studied in practical conditions the package and content of the can in the case of two different food stuff in cans fabricated with different tins and lacquers all for to compare the protection abilities of several lacquer systems and for extending the shelf life.

We used for the experiments the following types of tin lacquered cans: welded cans (3 pieces) Ø 99x48; pressed cans (2 pieces) Ø 73x30. For sealing these cans we used : Ø 99DV caps and Ø 73DV caps all lacquered in the corresponding lacquering systems.

We used the following lacquers: WHITE PL- inner protection white lacquer of epoxyphenolic type; HE GOLD – inner protection yellow lacquer epoxyphenolic type and PL GOLD – inner protection yellow lacquer of modified epoxyphenolic type.

The lab analysis of the lacquers observed:

- the global migration of the components in the food simulants A, B and D and the organoleptic exam of the samples and liquids after extraction:

- the specific components migration in the simulant A : the bisphenol A content (BPA) by two methods: UV-VIS spectrometry and gas chromatography coupled with mass spectroscopy GC-MS;

-the heavy metals migration from the package in the simulant B by atomic absorption spectroscopy (AAS) with graphite oven.

The checking of the quality of the cans was led on a relevant lot taken from a meat products manufacturer production (pork meat in natural juice and fois gras).

The filling and sterilization of the cans was led according to the specificity of the product. The can taken from the manufacturer according to STAS 3730-92 were stored in the conditions specified for the product. The canned products were regularly analyzed from

the organoleptic, physico-chemical and microbiological point of view during the entire storage period.

Table 1. The lacquer systems and products analyzed

Cans dimensions (mm)	Ø 99x48 welded cans (3 pieces)	Ø 73x30 pressed cans (2 pieces)
Product	Pork meat in natural juice	Fois gras
Lacquer system	Inner and outer side : WHITE PL lacquer	Inner side: WHITE PL lacquer
		Outer side: PL GOLD lacquer
	Inner side: HE GOLD lacquer	Inner side: HE GOLD lacquer
	Outer side: PL GOLD lacquer	Outer side: PL GOLD lacquer

The characteristics of the lacquer layer:

The analysis of the inner lacquer layer of a representative group of opened cans was done piece by piece and compared with the products standard conditions. The layer's porosity was determined with the porosymeter for both the empty and the filled and emptied (after a while) cans and the after a certain time of storage. The initial lacquer layer characteristics and the porosity during the storage time are presented in table no. 3 and 4.

The organoleptic, physico-chemical and microbiological analysis of the two types of food products (pork meat and fois gras) canned in the metallic cans was periodically led after 12 months of storage.

3. RESULTS AND DISCUSSIONS

The results of the lab analysis of the used protection lacquers are presented in table 2.

From the analysis of the results presented in **table 2** we can conclude the followings:

- there were neither organoleptic modifications regarding the appearance and smell of the tubes nor of the extraction liquids;
- the values obtained for the components global migration in all extraction environments were below the admitted value of 60 ppm;

The results of the global migration tests and of the organoleptic examination were in accordance with HG nr. 1197/2002, that contains specific directives such as 72/2002/CE and amendments .

- the heavy metals releases were below the admitted values : the Pb was below 0,02 ppm, and the Cd was below the detection limit of the device 0,12 ppb (the values were in accordance with the limits imposed in the Order no. 975/1998- repealed in the present time).
- the specific migration of bisphenol A (BPA) by the 2 methods UV-VIS spectrometry and gas chromatography coupled with mass spectroscopy GC- MS indicated- as it was expected- substantial differences between the obtained values of the same analyzed water extract.

Table 2. The lab analysis of the cans protection lacquers

Lacquer	Simulant	Extraction conditions/ Extraction ratio	Organoleptic modifications	Global migration, ppm	Metals release, ppm		Phenolic derivates, (Bisphenol A), ppm	
			Sample/ Extract		Pb	Cd	UV-VIS	GC-MS
GOLD HE	A: distilled water	1h, 121°C/ 1:1	Unmodified / Unmodified	7,75	-	-	1,6	BPA: 0,60
	B: 3 % acetic acid solution	1h, 121°C/ 1:1	Unmodified / Unmodified	9,75	0,012	<LOD	-	-
	D: isooctane	24h, 40°C/ 1:1	Unmodified / Unmodified	4,75	-	-	-	-
ALB PL	A: distilled water	1h, 121°C/ 1:1	Unmodified / Unmodified	9,5	-	-	1,2	BPA: 0,30
	B: 3 % acetic acid solution	1h, 121°C/ 1:1	Unmodified / Unmodified	16,5	0,015	< LOD	-	-
	D: isooctane	24h, 40°C/ 1:1	Unmodified / Unmodified	7,0	-	-	-	-
GOLD PL	A: distilled water	1h, 121°C/ 1:1	Unmodified / Unmodified	24,0			2,2	BPA: 0,85
	B: 3 % acetic acid solution	1h, 121°C/ 1:1	Unmodified / Unmodified	28,0	0,007	<LOD		
	D: iso-octane	24h, 40°C/ 1:1	Unmodified / Unmodified	14,0				

The GC-MS method allows the precise identification and dosage of bisphenol A (BPA) according to the mass fragment 213 characteristic for the bisphenol. On the basis of the calculated areas from the spectrograms we calculated with a precision the BPA concentrations. The determined values by this method are strictly individualized for BPA.

The spectrophotometric method UV- VIS unlike the GC-MS method led to higher values caused probably by other phenolic derivates, isomers that produce a signal juxtaposition at the same wave length that has as consequence an amplification of the absorbent. For evaluating the BPA (bisphenol A) results under the 2002/72/CE Directive and amendments we took on account the specific migration limit (SML) of 0,6 mg/kg.

From all the lacquers analyzed only the PL GOLD protection lacquer presents an overpassed value of BPA. That is not very significant for this type of lacquer is used for the outer protection.

The analysis of the inner side lacquer layer characteristics is presented in table 3.

From the analysis of the inner appearance of the cans we observed that after 12 months of storage in the case of the product „Pork meat in natural juice" –yellow lacquer type there appeared spots of marbling while in the case of „Fois gras"- yellow lacquer type the marbling appeared more accentuated for the meat itself.

After 1 year of storage at the shelf the porosity of the lacquer layer hasn't changed significantly for the majority of the cans. The results fitted in the technological norms of maximum of 89 mA – for the meat cans in natural juice.

Table 3. The initial characteristics of the lacquer layer – meat products cans

Characteristics	Product/ Inner lacquer			
	Pork meat/ White lacquer	Pork meat/ yellow lacquer	Fois gras/White lacquer	Fois gras/ Yellow lacquer
Layer resistance at sterilization in: - solution B, C, D, E	appropriate		appropriate	
Gr/m^2 (dry lacquer)	8,2 – 8,36	10,76 – 12, 04	7,24 – 7,40	10,76 – 12, 04
Layer adherence: - before sterilization - after sterilization	good good		good good	

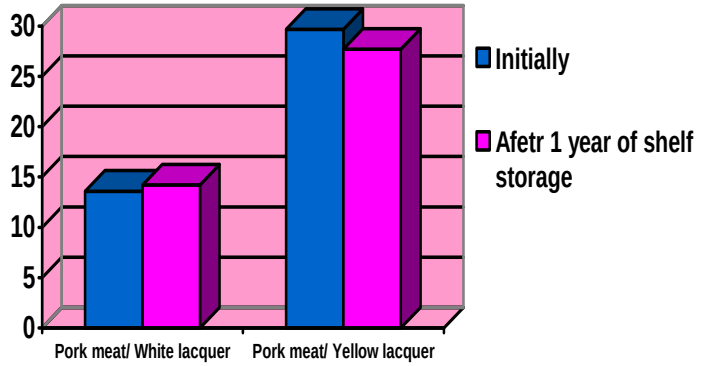


Fig.1. The checking of the porosity of the lacquer layer after the empting of the cans

After 12 months and 24 months of storage the organoleptic properties and most of the physico-chemical properties of the product „Pork meat in natural juice" didn't significantly changed in comparison with the initial moment for neither of the two lacquer types the exception being made by the easily hydrolysable nitrogen. During the storage after the 12 months and 24 months we observed slight increases of the easily hydrolysable nitrogen content in comparison with the initial moment for both lacquer types. These were due to the slightly degradation of the product during the storage and not to the lacquer layer itself. The metal content (Fe, Sn) of the product canned in metallic cans protected by white lacquer and yellow lacquers layers didn't significantly vary during the 12 months and 24 months of storage the product being in accordance with the requirements of STR 610-85 "Meat cans; fois gras and meat paste".

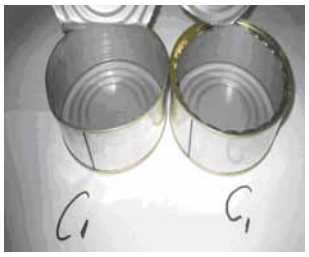
After 12 and 24 months of storage the organoleptic properties and most of the physico-chemical properties of the product „Fois gras" didn't significantly changed in comparison with the initial moment for neither of the two lacquer types the exception being made by the easily hydrolysable nitrogen.

During the storage after the 12 and 24 months we observed slight increases of the easily hydrolysable nitrogen content in comparison with the initial moment for the white

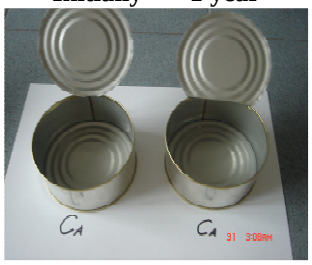
lacquer type. This was due to the slightly degradation of the product during the storage and not to the lacquer layer itself.

Table 4. The organoleptic analysis of the product «Pork meat in natural juice » (white lacquer)

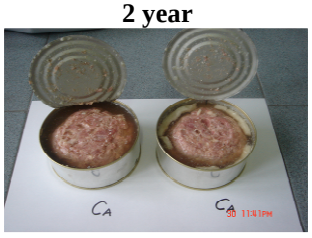
Characteristics	Results		
	Initially	**After 1 year of shelf storage**	**After 2 year of shelf storage**
Can's appearance: - outer side	Clean, hermetically sealed, with flat cap, unrusted, non deformed at the body or endings, without cracks or content leakage		
- inner side	The cap and metallic can don't have black or rust spots, or exfoliations and waving of the lacquer layer		
Content's appearance: - at 10^0C	The compact mass is formed by pieces of meat in jellified juice having at the surface a layer of fat.		
- at 40^0C	The meat pieces are obtained by chopping at the miner device with 20 mm holes, opalescent juice.		
Meat's colour	Specific for the boiled meat		
Fat's colour	White-yellowish	Yellowish	
Juice's colour	Yellow almost light brown		
Smell and taste	Pleasant, specific for the boiled meat and spices, without strange smell or taste		
Meat's consistency	Normal for the boiled meat		



Initially **1 year**



2 year



2 year

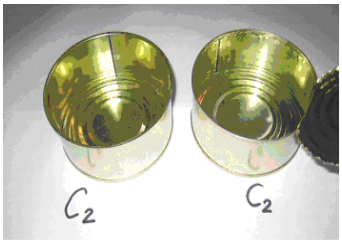
The results of the microbiological analysis

After the microbiological analysis of the meat cans in natural juice and of the fois gras cans we concluded the followings:

- the incubation of the cans by thermostat didn't lead to the appearance of cans that presented biological deformations or/ and leakages.
- after the cultivation in adequate environment of microorganisms we didn't registered modifications of the environment (environment altering, deposits or layers formation);
- the cans presented a good stability in time/ shelf life.

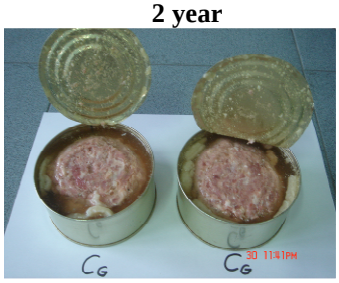
Table 5. The organoleptic analysis of the product «Pork meat in natural juice » (yellow lacquer)

Characteristics	Results		
	Initially	After 1 year of shelf storage	After 2 year of shelf storage
Can's appearance: - outer side	Clean, hermetically sealed, with flat cap, unrusted, non deformed at the body or endings, without cracks or content leakage		
- inner side	The cap and metallic can don't have black or rust spots, or exfoliations and waving of the lacquer layer	Traces of marbling on the can's wall	
Content's appearance: - at 10^0C	The compact mass is formed by pieces of meat in jellified juice having at the surface a layer of fat.		
- at 40^0C	The meat pieces are obtained by chopping at the miner device with 20 mm holes, opalescent juice.		
Meat's colour	Specific for the boiled meat		
Fat's colour	White-yellowish		White-yellowish
Juice's colour	Yellow almost light brown		
Smell and taste	Pleasant, specific for the boiled meat and spices, without strange smell or taste		
Meat's consistency	Normal for the boiled meat		



Initially **1 year**

2 year



2 year

Table 6. The physico-chemical analysis of the product "Pork meat in natural juice"

Characteristics	Initially		After 1 year of shelf storage		After 2 years of shelf storage	
	White lacquer	Yellow lacquer	White lacquer	Yellow lacquer	White lacquer	Yellow lacquer
Meat and fat, %	75,2	76,7	78,9	77,8	79,3	78.1
Fat , %	15,5	15,5	13,1	11,9	15,84	14,42
Sodium chloride, %	2,1	2,1	2,0	2,0	1,93	2,1
Easily hydrolysable nitrogen, expressed in ammonia mg/100 g	36,0	36,0	44,0	42,3	42,84	40,8
Proteins, %	20,20	20,20	20,79	19,81	20,5	20,24
pH	6,5	6,5	6,0	6,0	6,0	6,0
Metals , ppm:						
- iron	11,20	11,20	12,76	11,80	14,42	14,11
- tin	≤0,20	≤0,20	0,83	2,65	0,88	2,66

Table 7. The organoleptic analysis of the product "Fois gras" (white lacquer)

Characteristics	Results		
	Initially	After 1 year of shelf storage	After 2 year of shelf storage
Can's appearance: - outer side	Clean, hermetically sealed, with flat cap, unrusted, non deformed at the body or endings, without cracks or content leakage		
- inner side	The cap and metallic can don't have black or rust spots, or exfoliations and waving of the lacquer layer		
Content's appearance: - at 10^0C	Unctuous homogene paste that presents a thin liquid layer		
Colour	Pink-yellowish		
Smell and taste	Pleasant, characteristic with the flavour of the spices added without strange smell or taste		

Table 8. The organoleptic analysis of the product "Fois gras" (yellow lacquer)

Characteristics	Results		
	Initially	After 1 year of shelf storage	After 2 year of shelf storage
Can's appearance: - outer side	Clean, hermetically sealed, with flat cap, unrusted, non deformed at the body or endings, without cracks or content leakage		
- inner side	The cap and metallic can don't have black or rust spots, or exfoliations and waving of the lacquer layer	Traces of marbling on the can's wall	
Content's appearance: - at 10^0C	Unctuous homogene paste that presents a thin liquid layer		
Colour	Pink-yellowish		
Smell and taste	Pleasant, characteristic with the flavour of the spices added without strange smell or taste		

Table 9. The physico-chemical analysis of the product "Fois gras"

Characteristics	Initially		After 1 year of shelf storage		After 1 year of shelf storage	
	White lacquer	Yellow lacquer	White lacquer	Yellow lacquer	White lacquer	Yellow lacquer
Fat , %	21,7	21,7	22,0	20,0	21,33	21,15
Sodium chloride, %	1,7	1,7	1,7	1,7	1,81	1,75
Easily hydrolysable nitrogen, expressed in ammonia mg/100 g	18,0	18,0	23,1	14,3	21,81	14,1
Proteins, %	13,80	13,80	12,87	13,14	13,62	13,4
pH	6,0	6,0	5,5	5,5	5,5	5,5
Metals , ppm:						
- tin	-	-	-	-	5,30	5,30

CONCLUSIONS

The results of our studies were the following:

- the results of the lab examination respectively of the global migration tests and the organoleptic exam are in accordance with HG nr. 1197/2002, that contains specific directives such as 72/2002/CE and amendments.

- for evaluating the BPA (bisphenol A) release as a request of the 2002/72/CE directive we considered as more appropriate the GC-MS method that allows the accurate identification and dosage of bisphenol A (BPA) in comparison with the UV-VIS spectrophotometric method .

- the checking of the cans quality was led on a lot taken from a meat products manufacturer (pork meat in natural juice and fois gras).

- after 12 and 24 months of storage the organoleptic properties and most of the physico-chemical properties of the products („Pork meat in natural juice" and "Fois gras") didn't significantly changed in comparison with the initial moment for neither of the two lacquer types.

- from analyzing the inner appearance of the can we saw that at 12 and 24 months it presented all over it traces of marbling in the case of the product „Fois gras"- yellow layer type the phenomena being more accentuated than in the case of the other product.

- the metal content (Fe, Sn) of the product canned in metallic cans protected by white lacquer and yellow lacquers layers didn't significantly vary during the 12 and 24 months of storage the product being in accordance with the requirements of STR 610-85 "Meat cans; fois gras and meat paste".

- all the analyzed quality indicators are in accordance with the standard of the product.

- after the microbiological analysis of the meat cans we concluded that the cans have a very good shelf life/ time stability.

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ANALYSIS AND QUANTIFICATION OF TRANS-RESVERATROL IN WINE FROM MUNTENIA AND OLTENIA REGION, ROMANIA

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Abstract. *Known for its several beneficial effects on human health (antioxidant, antibacterial, cardioprotective, antitumor, estrogenic, antiplatelet, anti-inflammatory, etc), the first supplement known to activate a longevity gene, resveratrol, is one of the major active compounds of stilbenes. It is a phytoalexin found in grapes, grape products, wine, peanuts, cranberries, strawberry, and some other botanical sources, in variable amounts, both as free and as piceid.*
The aim of this work is the development of a high performance liquid chromatographic (HPLC) method for the determination of trans-resveratrol in wines from Muntenia and Oltenia region delimited appellation (Romania). The method permits direct injection of the sample followed by PDA detection, revealing to be time-saving and overcoming the need of sample pretreatment steps. Detection limit was 0.008 mg/L. Global uncertainty associated with the results, according to EURACHEM/CITAC rules was 23.5%.
Resveratrol content was determined for 24 Romanian AO (appellation of origin) wines; the concentration found ranging between 0.132 and 6.589 mg/L. The wines from Muntenia region have grater content of trans-resveratrol than wines from Oltenia region. The levels of trans-resveratrol were clearly higher in the red wines compared to the white wines (never exceeding 0.159 mg/L).

Keywords: trans-resveratrol, wines, HPLC

1. INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene) (Fig. 1) is a phytoalexin of a phenolic nature, belonging to the stilbene family. Naturally it exists in two isomeric forms (cis and trans) and as piceid, the 3 β-glucoside of resveratrol. It occurs in grapes, grape products and some other botanical sources (peanuts, soy, tea), in variable amounts, both as free and as piceid [1].

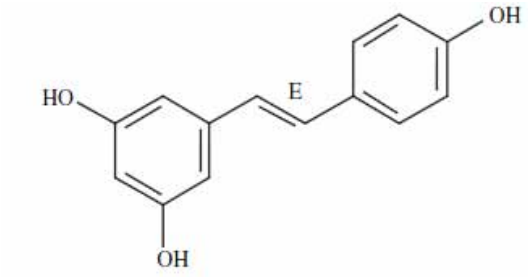

Figure 1. Structure of trans-resveratrol

The presence of trans-resveratrol in wine is an important qualitative parameter due its several beneficial effects on human health: has anti-oxidant and anti-inflammatory activity, is in relation with anti-initiation, anti-promotion and anti-progression activities in malignant tumors and is recognized as a chemotherapeutic agent in humans [2].

Trans-resveratrol plays also an important role in the organoleptic characteristics of wine, conferring astringency and structure to the beverage by formation of complexes with the proteins of saliva [3].

Wine, particularly red wine, is the main source of introduction of trans-resveratrol in the human diet. In grape berries, resveratrol synthesis is primarly located at the skin cells and it is absent or low, in the fruit flesh [4].

Red wine generally contains higer amounts of trans-resveratrol than white wine. This is presumably due to the longer extraction time during contact between grape skin and juice in the production of red wine [5]. The variety of grapes also plays an important role in resveratrol synthesis which may be genetically controlled. Resveratrol concentration increased during fermentation of the skins but the amount extracted was depended on the variety and enological conditions [6].

Traditional methods to determine polyphenols in natural extracts are usually performed by high performance liquid chromatography analysis (HPLC) and spectrophotometric measurements [7]. Methods based on gas chromatography and gas chromatography-mass spectrometry (GC-MS) have been proposed for trans-resveratrol. However, derivatization is required prior to GC analysis of this analyte to enhance volatility; this time consuming procedure may result in some trans to cis isomerization of resveratrol. The development of LC-MS techniques are nowadays the best analytical approach for the analysis of this kind of compounds in grape extract and wine, although this hyphenated techniques is very expensive and consequently not widely used in routine laboratories of wine industry [8].

This work presents a reversed phase RP-HPLC method for the determination of trans-resveratrol in wine samples. Analyses were carried out by direct HPLC injection of the sample in solvent elution. A group of 24 wines from Muntenia and Oltenia Romanian regions, different cultivars and vintage year was analysed by HPLC-PDA, in order to assess the influence of these parameters on the trans-resveratrol concentration.

2. MATERIALS AND METHODS

Reagents and chemicals - The trans-resveratrol standard (99% GC) was purchased from Sigma-Aldrich. Methanol, acetonitrile and glacial acetic acid (100% p.a.) were HPLC grade and obtained from Merck. Twice distilled and demineralised water produced by TKA systems was used for preparation of the aqueous solutions.

Standard solutions - A stock solution of 20 mg/L of trans-resveratrol prepared in a 12% alcoholic (aqueous) solution. The solid standard was initially dissolved in a minimum volume of ethanol, before the addition of water, in order to guarantee a complete dissolution. Immediately prior to analysis, a set of standards with 0.25, 0.5, 1, 1.5, 2, 3, 5 and 10 mg/L were prepared from the stock solution, diluted in a 12% alcoholic (aqueous) solution. Special care was taken in relation to the degradation of the standard solutions, keeping them protected from air and light exposure.

Sample handling - In this study 24 wine samples (10 white and 14 red) from Muntenia and Oltenia region were analyzed. The analyzed red wine varieties were Feteasca Neagra, Cabernet Sauvignon, Merlot, Pinot Noire and those of white wines varieties were Feteasca Regala, Tamaioasa Romaneasca, Sauvignon Blanc, Feteasca Alba, Riesling Italian and Muscat Ottonel. All samples were filtered through 0.45 µm filters prior to analysis and kept protected from light at 4 °C

HPLC analysis

The chromatographic analysis was carried out with a Thermo Finnigan Surveyor Plus chromatograph equipped with a photodiode array detector at 310 nm, Surveyor autosampler, Surveyor LC Pump (Quaternary gradient). Data analysis was done using the Chrome Quest Chromatography Workstation.The analytical column aquasil C18, 250×4.6 mm with the sorbent particle size of 5 µm. The mobile phase mixed of water:acetonitrile:acetic acid = 70:29.9:0.1 has flown through the system at a rate of 1 ml/min. Samples of 20 µl volume were injected into the analytical column. All analyses were carried out at ambient temperature. Quantification was based on the peak area, using the external standard method.

3. RESULTS AND DISCUSSIONS

Validation studies

Calibration curve was performed by analysis in duplicate of eight standards of trans-resveratrol covering the range 0.25-10 mg/L. The constructed calibration curve shoved excellent linearity (correlation coefficient: 0.9991) (fig. 2).

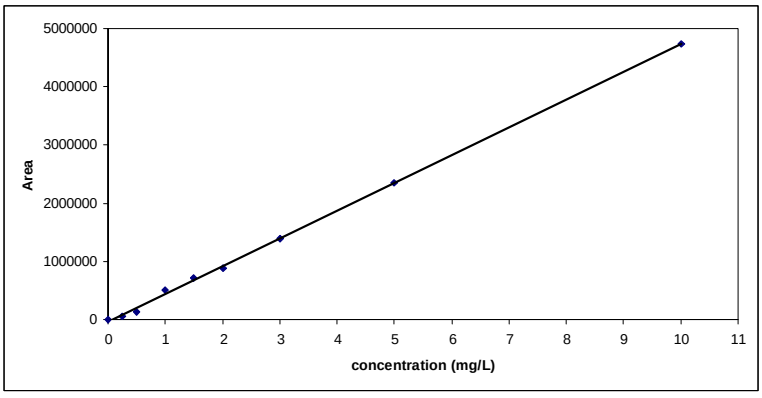

Figure 2. Calibration curve for trans-resveratrol

The linearity of the detector response for the prepared standards was assessed by means of a linear regression analysis regarding the amounts of each standard introduced in the loop of the chromatographic system and the area of the corresponding peak on the chromatogram.

Detection and quantification limits were calculated by measuring the response of analytical background by running six blanks (12% alcoholic solution), using the maximum sensitivity allowed by the system and calculating the standard deviation of this response

(STD). The detection limit, DL was estimated by multiplying the STD by a factor of 3, and the quantification limit, QL was defined as 10 times the STD. The values obtained were 0.008 mg/L for DL and 0.026 mg/L for QL.

The precision or the degree of reproducibility is expressed as the coefficient of variation (CV). Precision was determined by performing six replicate analyses of the same sample on six different days. The value obtained for precision was 9.8 % for direct injection of the sample in chromatographic system.

The uncertainty was estimate according EURACHEM/CITAC. This estimation accounts for the most significant sources of uncertainty that are thought to interfere in the final result. In this work, the contributions to uncertainty were ascribed to: the uncertainty in the preparation of standards, the uncertainty of the calibration curve and the uncertainty in the precision of the method. The global uncertainty was estimated at 23.5 % for concentration above 0.25 mg/L.

Quantification of trans-resveratrol in Romanian wine

The determination of the trans-resveratrol alone proved to be accurate using a simple procedure, without sample pre-treatment other than filtration, allowing for a less than 15 minute analysis run time.

However, the chromatograms of the standards are free from interferences, with the peak of trans-resveratrol clearly separated (Fig. 3a). Regarding the wine samples it is noticeable that in the red wine (Fig. 3b) the interferences are more significant than in white wines (Fig. 3c), in spite of the lower amounts found in the latter samples.

In the work of Ratola et all [9], Portuguese red wine from different regions had trans-resveratrol content from 0.55 to 1.24 mg/L and white wines had values ranging from not detected to 0.19 mg/L. Other studies report trans-resveratrol content ranging from 0.508 to 2.534 mg/L for greek wines [10] and values ranged from 0.25 to 8.87 mg/L for Italian wines [11].

The amount of trans-resveratrol in the different analyzed wines varied considerably depending on the grape variety, environmental factors in the vineyard and wine-processing techniques. In our study, the occurrence of trans-resveratrol was confirmed in all of the analyzed 14 red wine samples, with concentration ranging between 0.025 and 6.598 mg/L (Table 1). For the white wine, 5 of the 10 tested revealed the presence of the compound at concentration between 0.132- 0.159 mg/L (Table 2).

It is known that trans-resveratrol is produced by grape berries in response to fungal infection and UV irradiation [12]. Thus, it is possible that sun exposure of grapes grown at Muntenia region may be a factor in increased resveratrol level compared with the grapes from Oltenia region. In addition, the red grape varieties Feteasca Neagra may be genetically richer in this compound than other varieties.

Graphical representation of mean concentration of trans-resveratrol found in this research, depending on the red wine variety show that the content of trans-resveratrol decreased in order Feteasca Neagra, Merlot, Pinot Noire, Cabernet Sauvignon (Fig. 4). Concerning white wine, higher concentration of trans-resveratrol was found in Muscat Ottonel variety.

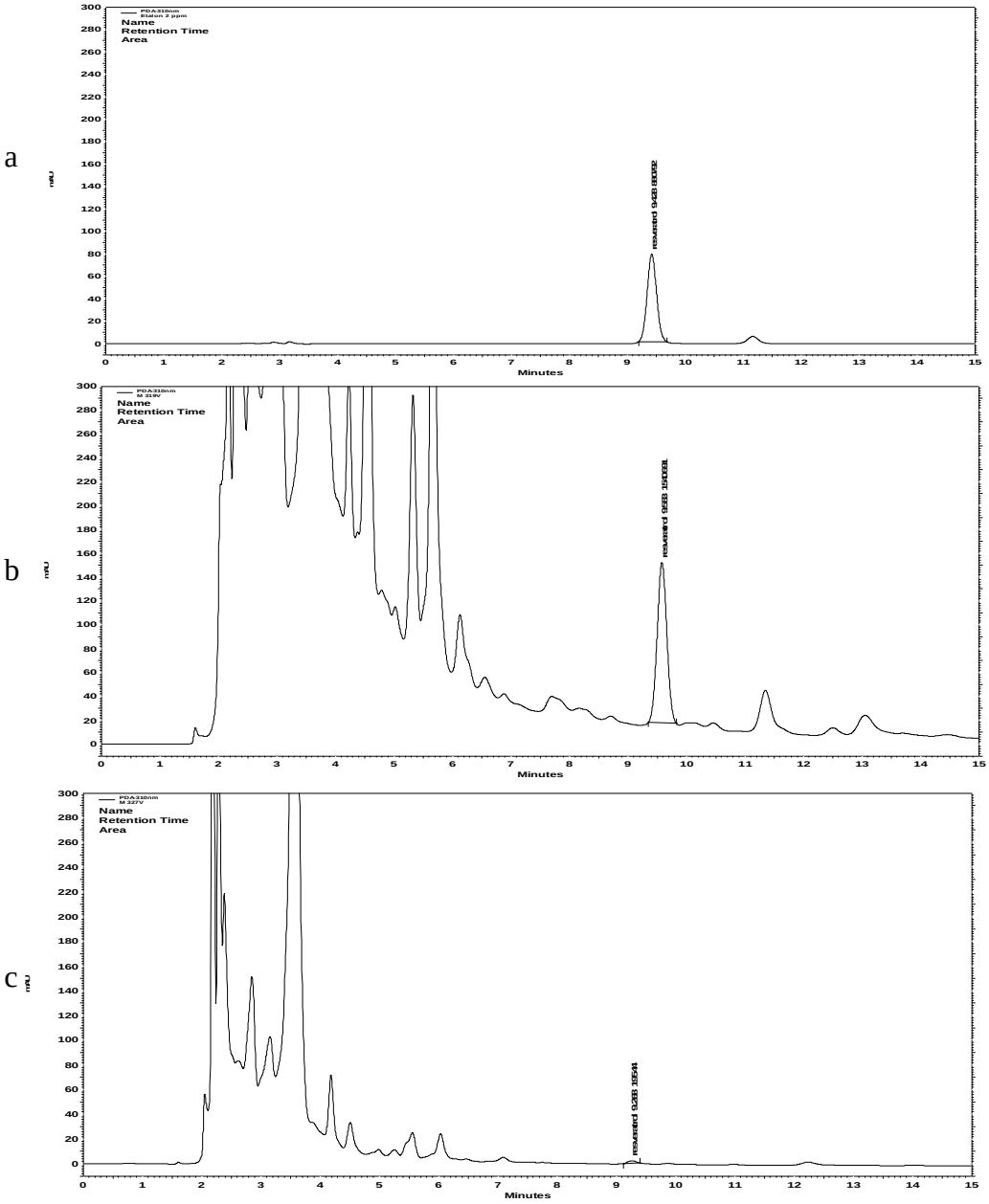

Figure 3. Chromatogram for: (a) a trans-resveratrol standard solution; (b) a red wine sample; (c) a white wine sample

Table 1 Trans-resveratrol content of Romanian red wines grouped on varieties, geographical origin and harvest year

Variety	Region	Harvest year	Trans-resveratrol (mg/L)
Feteasca Neagra	Muntenia (Valea Calugareasca)	2008	4.363
	Oltenia	2008	1.501
	Muntenia (Valea Calugareasca)	2009	6.589
	Muntenia (Dealu Mare)	2009	0.256
	Oltenia (Olt)	2009	1.717
Cabernet Sauvignon	Muntenia (Tohani - Dealu Mare)	2008	0.262
	Muntenia (Urlati)	2008	0.499
	Oltenia (Mehedinti -Vinju Mare)	2008	0.025
	Oltenia - (Mehedinti -Vinju Mare)	2009	0.149
Merolt	Muntenia (Tohani - Dealu Mare)	2008	3.307
	Muntenia (Tohani – Dealu Mare)	2008	2.771
	Oltenia - (Mehedinti -Vinju Mare)	2008	1.621
Pinot Noire	Muntenia (Valea Calugareasca)	2008	2.369
	Muntenia (Valea Calugareasca)	2009	1.198

Table 2. Trans-resveratrol content of Romanian white wines grouped on varieties, geographical origin and harvest year

Variety	Region	Harvest year	Trans-resveratrol (mg/L)
Feteasca Regala	Muntenia (Dealu Mare-Pietroasele)	2008	n.d.
	Muntenia (Dealu Mare-Pietroasele)	2009	0.132
Tamaioasa Romaneasca	Muntenia (Dealu Mare-Pietroasele)	2008	n.d.
Sauvignon Blanc	Muntenia (Tohani – Dealu Mare)	2008	0.074
	Muntenia (Tohani – Dealu Mare)	2009	0.138
Feteasca Alba	Muntenia (Tohani – Dealu Mare)	2008	n.d.
	Muntenia (Tohani – Dealu Mare)	2009	n.d.
Riesling Italian	Muntenia (Valea Calugareasca)	2008	n.d.
	Muntenia (Valea Calugareasca)	2009	0.141
Muscat Ottonel	Muntenia (Urlati)	2009	0.159

n.d. not detected (detection limit = 0.008 mg/L)

4. CONCLUSIONS

This work enabled the implementation of an easy and reliable methodology for trans-resveratrol analysis in wine. The use of direct injection in HPLC with PDA detection is a practical method of analysis used by many laboratories. Validation studies for the method show a detection limit of 0.008 mg/L, quantification limit of 0.026 mg/L and a global uncertainty of 23.5%.

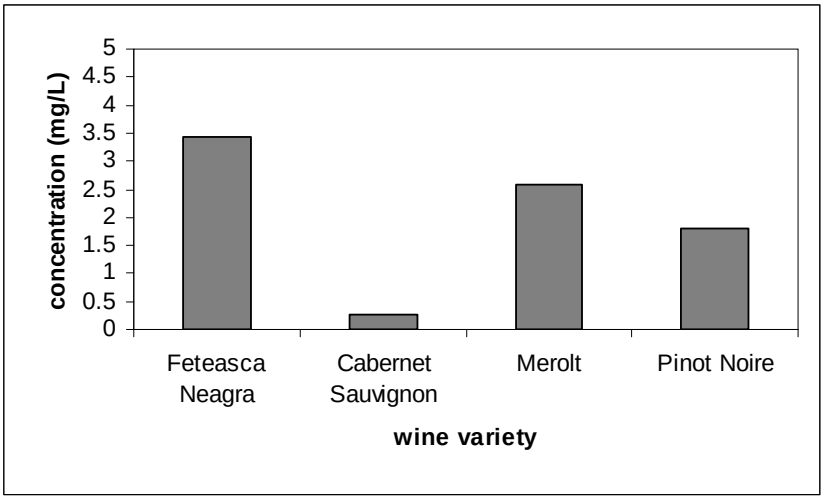

Figure 4. Representation of experimental average concentration of trans-resveratrol depending on the red wine variety

As expected, the levels of trans-resveratrol are clearly higher in red wines, most of the white wines being actually under the detection level of the method (0.008 mg/L). The level of trans-resveratrol found here are comparable to those found by other authors [9-11], which demonstrates the suitability of this very simple analytical technique.

For a public health point of view, the values obtained may suggest that the consumption of wine from Feteasca Neagra variety represent a reliable source of health.

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CAROTENE CONTENT FROM MILK SAMPLES OBTAINED IN DIFFERENT FEEDING CONDITIONS

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Abstract. *Carotene is important in human health and nutrition, because is a precursor to vitamin A. In spite of lower percentage in milk, carotene is involved in the sensorial properties of dairy products through their colorant and antioxidant properties. The aim of this research was to study the variability of carotene content according to farm feeding practices. Carotene content was determined using UV-VIS 550 spectrophotometer. Milk samples were analyzed from physico-chemical point of view. The metal content (Cu, Fe and Zn) of each sample was determined by Atomic Absortion Spectrophotometer (Analyst 400). The obtained results showed a variation of beta carotene content according to the feeding mode of cows.*

Keywords: milk, carotene, spectrophotometer, Atomic Absorbtion

1. INTRODUCTION

Carotenoids are notable for their wide distribution, structural diversity, and various functions. Animals are incapable of carotenoid biosynthesis, and hence depend on dietary carotenoids, which are selectively or unselectively absorbed, converted to vitamin A.[4]

Even it is obviously in several countries that carotene contribution in human diet is coming in large percentage from carrots, as well as from other fruits and vegetables, a smaller concentration is available from diary products, giving a better position to milk in consumer acknowledge. More, the carotenes may play a rol in stabilising the oxidable compounds of milk. Despite their benefic roles, a special attention should be paid to carotene transfer from feeding to cow milk.[3]

Data from literature shows about 10 identified carotenes in feed (ex. Carotene, xantofile), their concentration depending on development status and their conservation period. The sensitivity of β-carotene during rumination is depending a lot of the feeding source. From the class of ruminants, only cattle accumulates high concentration of carotenes, especially β-carotene, most probably due to the low efficiency of vitamin A synthesis in enterocytes. Carotenes from cow milk are mostly β-carotenes *trans* and, in lower concentration, lutein. In milk, the concentration varies more in the case of β-carotene than for retinoic those plasmatic concentration is well fixed. The β-carotene concentration in milk is depending on ditetic suplements used. The animal and the factors related to him is affecting the milk production (i.e race).[2]

In this article the presented research results are concerning the level of β-carotene from milk of the cows grew in plain and mountain areas, differentiated fed due to season.

2. MATERIALS AND METHODS

2.1 SAMPLES OF MILK

For completing the experiments, milk samples from two areas have been analyzed: from plain area and from mountain area from cows fed with different types of feed. 11 milk samples of cows fed both summer and winter periods from plain area of IBNA Balotesti have been analyzed. From the mountain area of Blana Bucegi milk samples of cows grazing on different pasture have been analyzed.

Table 1. Milk samples used in experiments

Sample cod	Feeding mode	Origin
P1,P2, P3	combined fodder, hay - alfalfa, corn silage	IBNA Balotesti
A1,A2,A3	natural pasture fertilized	experimental fields Blana Bucegi (1800 m)
C1,C2,C3	natural pastures, limed, chemical fertilised, padocked	
D1,D2,D3	sown pasture, limed, chemical fertilised, padocked	
E1,E2,E3	natural pastures near the experimental field	
TI,T2,T3	natural pastures from the experimental field	
P4,P5,P6,P7,P8,P9,P10,P11	combined fodder, green fodder	IBNA Balotesti

Milk samples A1..3; C1..3 ; D1..3; E1..3; T1..3 have been taken in august from 15 cows fed on 5 different pastures each of 0,75 ha. On each pastures 3 cows have been fed about 85 days on summer time. The pastures differ on type, level and fertilization level as well as the exploiting way. Before analysis, the samples have been frozen at -18^0C.

2.2 METHODS

- UV-VIS

The concentration of β-carotene from the considered milk samples has been determined with UV-VIS 550 spectrophotometer. The milk samples have been thawed, heated at room temperature, homogenized and then analyzed. Saponification of fat from milk has been done at cold with hydro-alcoholic solution of potassium hydroxide and carotene extraction was done with n-hexane. The extract purification has been done on chromatographic column (activated aluminum oxyde and anhydrous sodium sulphate). The β-carotene extract colouring intensity in hexane has been measured with the spectrophotometer (λ=450 nm, the reference sample was n-hexane).

The content of β-carotene, in µg at 100 ml of product, was calculated with the following formula:

$$\beta\text{-carotene} = \frac{C \times V_1}{V} \times 100 \quad [\mu g/100ml]$$

of which:

C – the concentration of β-carotene, red on calibration curve in µg/ml

V_1 – the extract volume of β-carotene in hexane, in ml

V – the sample volume for determination, in ml

- The physico-chemicals characteristics of the considered milk samples have been determined accordingly with standardized methods shown in table no. 2

Table 2. The Standards used at physico-chemical analysis of milk samples

Quality indicators	M.U.	Method
Fat	%	STAS 6352/1-88
Dry substance	%	AOAC 990:20/2005
Protein	%	SR EN ISO 8968-3:2007
Acidity	^{0}T	SR ISO 6092:2008

- The determination of heavy metals and mineral elements

To do the determination, an *AAnalyst 400 Perkin Elmer* atomic absortion spectrophotometer has been used (air-acetylene, with corection of absortion (lamp D2) to analyse metals and mineral elements: Zn, Fe, Cu, Ca, Mg)
The recommended wave lenght for the above mentioned elements are:
Cu = 324,8 nm; Fe = 248,3 nm; Zn = 213,9 nm; Ca = 422,7 nm; Mg = 285,2 nm.
The analyte concentration for each element was determined with the sampling curve:
Absorbance = f (concentration), completed with the respective sampling solutions
Mineral element Determination: sampling curve for Mg was completed between 0 and 0,5 ppm, and for Ca between 0 and 5 ppm.
Metal Determination: sampling curve for Cu was completed between 0 and 4 ppm, for Fe between 0 and 3 ppm and for Zn between 0 and 0,75 ppm.

3. RESULTS AND DISCUSSIONS

- **Concentration of β-carotene**

To quantify the level of β carotene from the considered milk samples, we did a calibration curve between 0 and 2 µg β carotene/ml. (the linearity coefficient R^2=0.9994).

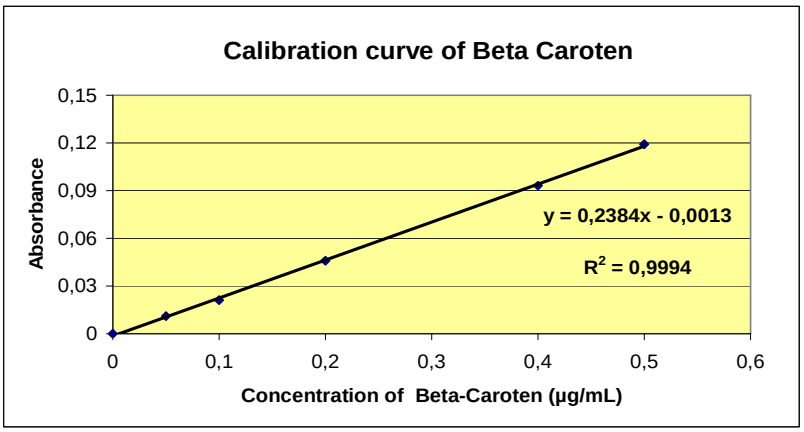

Figure 1. Calibration curve of β carotene

The β-carotene content, in µg at 100 ml of product, determined for the considered samples, is presented in graphic below.

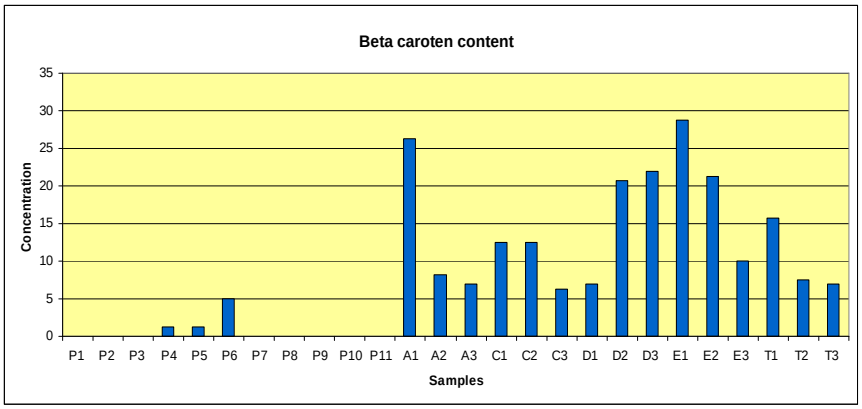

Figure 2. The β-carotene content

In the case of milk samples (P1,P2,P3) taken from the cows fed with winter feed (combined fodder, hay - alfalfa, corn silage) no detection of the β-carotene content. This results can be explained by the fact that this feed has a low β-carotene content (0-0,08 mg β-carotene in 100 g of feed) and after cow metabolism it was not further found in milk.[1] In the case of the milk from cows fed on summer time (combined fodder, green fodder) at IBNA Balotesti, the β-carotene content has been detected at samples P4,P5,P6, between 1,25-5 µg β-carotene per 100 ml. After the study was completed, we noted that β-carotene accumulation in cow milk is influenced both of the feed β-carotene content and the each cow metabolism.

Largest values of the β-carotene concentration have been recorded in the milk samples taken at Blana Bucegi, where the cows have been fed with green feed on open space. In this case, the β-carotene content varied between large limits of 6,25 – 28,75 µg β-carotene per 100 ml. The β-carotene concentration is referring to individual milk samples, the minimum value is referring to the milk sample from cow no. 3 (C3), fed on parcel C (natural pastures, limed, chemical fertilised, padocked). The maximum value is referring to β-carotene content of the milk sample from cow no. 1 (E1), fed on natural pastures closed to experimental area (pasture E).

Besides the variation of β-carotene content depending of the pastures where cow was fed, there are differences between β-carotene content of milk samples from cows which were fed on the same pastures (6,88-15,63 µg β-carotene per 100 ml for the cows fed on the natural pasture within the experimental area, pasture T). These results can be explained due to the crumbling degree, digestion and metabolisation degree of the feed within each animal.

- **Physico-chemical characteristics**

The considered milk samples have been analyzed also from physico-chemical point of view and the results are presented in the graphic below.

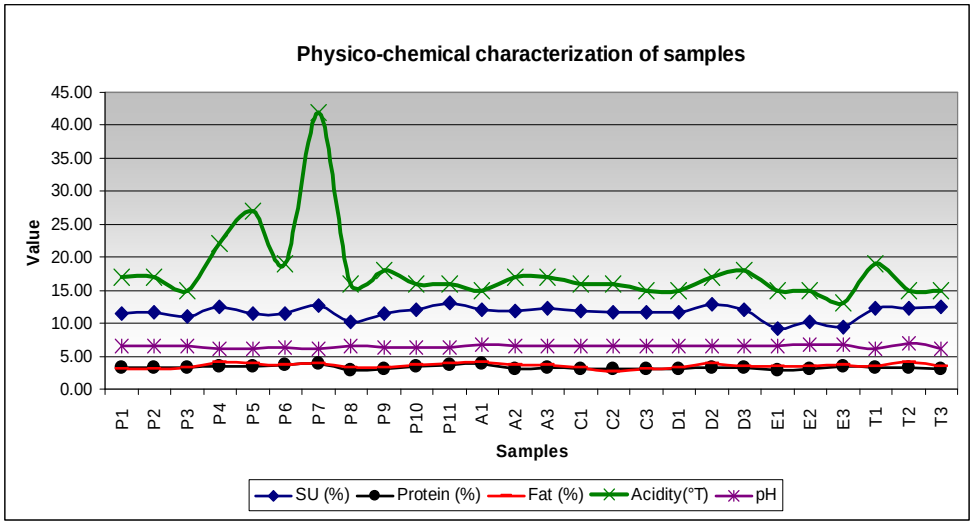

Figure 3. The variation of physico-chemical properties of samples

Accordingly with the obtained results, the protein content of milk samples, taken from the considered cows, varied between 2,88-3,79%. The minimmum value has been registered in the case of sample P8 from a cow fed on plain area with green feed and combined fodder. The maximum value is referring to sample P7.

The content in fat of milk samples from considered cows, varied between 2,68-4,16%, the minimmum value has been registered in the case of a milk sample from cow no. 2, fed on a natural pastures, limed, chemical fertilised, padocked. The maximum value has been registered in the case of a milk sample P4 from a cow fed on plain area with green feed and combined fodder.

The variation of metal content (Cu, Fe,Zn) and mineral elements (Ca,Mg) for the considered milk samples is presented in the table below.

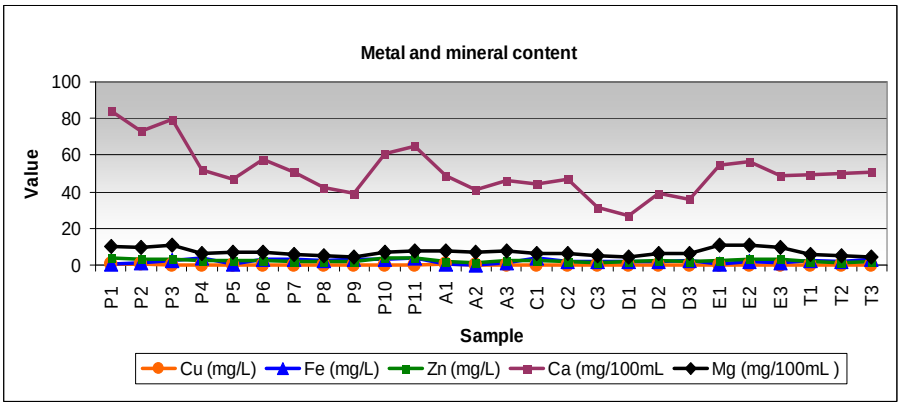

Figure 4. The variation of metal and mineral content

As it is shown in the graphic above, the iron content of the considered milk samples varies in large limits: 0,139 mg/l-3,889 mg/l, the minimmum value at milk sample A2, from cow no. 2, grew on pastures A (natural pasture last time fertilised on 2010). The maximum value has been registered in the case of milk sample C1, from cow no. 1, fed on parcel C (natural pastures, limed, chemical fertilised, padlocked).

Concerning the calcium content, the biggest value has been registered in the case of sample A1, from cows fed with combined fodder, hay - alfalfa, corn silage, on plain area.

4. CONCLUSIONS

1. Following the study, we noted that β-carotene accumulation in cow milk is influenced both β-carotene content in feed and each cow metabolism.
2. The milk samples taken from Blana Bucegi, where the cows have been fed with green feed on open space, have been presented the biggest concentrations in β-carotene, in between 6,25 – 28,75 µg β-carotene on 100 ml.
3. The iron content of the considered milk samples varied between 0,139 mg/l-3,889 mg/l. The minimmum value has been registered in the case of milk sample from a cow which has been fed on a natural pastures (last time fertilised on 2010, sample A2) and the maximum value has been registered in the case of a milk sample from a cow fed on natural pastures, limed, chemical fertilised, padocked (sample C1). The milk from cows fed with combined fodder hay - alfalfa, corn silage, grew on plain area, has been presented the largest content in calcium: 84,15 mg/100 mL (sample A1).

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RESEARCH ON MYCOTOXIN CONTENT OF *TRITICUM AESTIVUM* IN ROMANIAN SOUTH CROPS

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Abstract. *In this study, there have been analyzed 69 wheat samples (Triticum aestivum, Dropia variety) from 8 different regions situated in southern Romania; samples were provided in 2010 right after harvesting.*

For these samples were determined microbiological analysis (total number of germ, yeasts and molds), mycotoxin content (deoxynivalenol – DON, zearalenone – ZEA) and also physical-chemical tests (percentage of impurities and foreign bodies, number of moldy grains).
Following the interpretation of results it was found that in all wheat samples studied, the mycotoxin content was below the maximum levels allowed for unprocessed cereals established by CE no. 1881/2006 regulation, except for Teleorman county were 2 samples exceed the limit for ZEA content.

There were samples in which mycotoxin content was not detected: 92.7% of samples analyzed for ZEA, respectively 34.8% for DON content, the values being below the limit of detection of the method used for analysis.

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Keywords: ***Triticum aestivum*, Dropia variety, deoxynivalenol, zearalenone**

1. INTRODUCTION

Mycotoxins are secondary metabolites produced by fungi, with different and complex chemical structures and common characteristics, such as: resistance to thermal degradation, persistence during processing of products. Once formed mycotoxin will reach the final consumer products.

Wheat can be contaminated with mycotoxins produced by a variety of fungi, in particular by *Fusarium* species [1]. Growth of these fungi and formation of mycotoxins can occur, depending on some conditions, such as: crop growth, harvest, transport, storage (fungi multiply when relative humidity is 80 – 85%, and temperature is high, above 26^0C) and processing [2].

The most important *Fusarium* mycotoxins that can frequently occur at biologically significant concentrations in cereals are: fumonisins, zearalenone (ZEA) and trichothecenes: deoxynivalenol (DON), nivalenol and T-2 toxin. Mycotoxins DON and ZEA are produced by *Fusarium graminearum*, *Fusarium culmorum* and *Fusarium crookwellense*.

DON (also called vomitoxin) is the most important trichothecenes because of its high incidence in cereals, including maize, wheat, barley, oats, but it is not one of the most acutely toxic of this group of mycotoxins [3].

ZEA may be present in cereal crops in cooler and moist regions worldwide.

Mycotoxins not only cause serious damages in yields and quality of grains, but also can induce a variety of adverse health effects in humans and animals, depending on contamination levels of the products. Due to the increased concern about protection of the consumer, mycotoxins are monitored in the European Union with official regulations [4]. Thus, mycotoxins are strictly regulated in the Commission Regulation EC no. 1881/2006 [5]; for instance, maximum level for DON in unprocessed wheat is set to 1250 □g/kg, respectively, 100 □g/kg for ZEA.

In this work, a monitoring of wheat samples (Dropia variety) from the point of view of mycotoxin content has been performed in some regions from southern Romania.

2. MATERIALS AND METHODS

Samples preparation

The selected samples for analysis were wheat *Triticum aestivum*, grown in southern Romania, collected from local producers in 2010. There have been analyzed 69 wheat samples coming from 8 different geographical regions: Dolj (13 samples), Călărași (12 samples), Olt (8 samples), Dâmbovița (4 samples), Buzău (9 samples), Brăila (4 samples), Mehedinți (3 samples) and Teleorman (16 samples). All the samples belonged to Dropia variety, which is preponderant in south region for bakery wheat. In this study, the samples were analyzed right after harvesting.

Samples preparation consists in grinding of almost 500 g wheat in order to obtain a homogeneous powder.

Extraction procedure for ZEA mycotoxin: 25 mL mixture methanol / water (70/30) is added to 5 g ground sample, stirred (at 180 rpm, 10 minutes, room temperature), followed by extract filtration on Whatman no.1 paper. The same procedure for DON extraction, except that only water is used as solvent.

Methods used for analysis

Detection and quantification of mycotoxin content was determined using ELISA type immuno-enzymatic method (Ridascreen Zearalenon kit for ZEA, respectively, Ridascreen Deoxynivalenol kit for DON – producer R–Biopharm, Germany). Optical density are read (at 450 nm) with Sunrise spectrophotometer (Tecan, Austria), equipped with Rida®SOFT Win program (R–Biopharm, Germany). Two repetitive samples have been used and the average media of these samples has been employed in data analysis.

Procedure for microbiological analysis is based on counting colonies of microorganisms obtained on solid media, by incubation. Thus, microbiological indicators, such as total number of germ on Plate Count Agar and number of yeasts and molds on Glucose – Chloramphenicol Agar were determined.

The determination of impurities was carried out according to SR ISO 7970:2001 – Wheat (Triticum aestivum L.). Specifications – which refers to the average quality of wheat [6].

3. RESULTS AND DISCUSSIONS

The microbiological analysis: total number of germ (NTG), yeasts and molds (YM), mycotoxin content (DON, ZEA) and percentage of impurities and foreign bodies and moldy grains are presented in Table 1 for the wheat samples analyzed in all the 8 counties.

For 33.3% of the samples analyzed, impurities and foreign bodies content exceeds the maximum accepted percentage of 15% due to the percentage of kernels affected by pests (bug especially) and sprouted or fungal contamination.

In the same county, Dropia variety had different mycotoxin values, varying from below the limit of detection to higher values. For example, in Teleorman county DON content varied from not detected values to 279.94 g/kg. Also, same situation in Dolj, Buzău and Brăila. This can be due to the local environmental condition (soil conditions, meteorological conditions, harvesting agricultural technologies).

Table 1. Results obtained for wheat samples of 2010 crop for different counties.

Locality	NTG (ufc/g)	YM (ufc/g)	DON (□g/kg)	ZEA (□g/kg)	Impurities (%)	Moldy grains (%)
Dolj county						
Mischii	$1.20 \cdot 10^6$	$1.70 \cdot 10^4$	11.87	n.d.*	6.33	0
Teslu	$4.80 \cdot 10^5$	$2.00 \cdot 10^4$	31.01	n.d.*	4.53	0.1
Scaesti	$4.50 \cdot 10^5$	$7.30 \cdot 10^3$	11.97	n.d.*	13.37	0
Ciupercenii-Noi	$3.20 \cdot 10^5$	$1.00 \cdot 10^3$	12.96	n.d.*	4.08	0
Macesu de Sus	$8.20 \cdot 10^4$	$4.00 \cdot 10^3$	43.94	n.d.*	1.24	0
Daneti	$2.00 \cdot 10^6$	$4.50 \cdot 10^4$	554.39	n.d.*	32.42	0.33
Plenita	$1.50 \cdot 10^5$	$1.00 \cdot 10^4$	n.d.*	n.d.*	4.8	0
Motatei	$4.00 \cdot 10^4$	$9.00 \cdot 10^3$	6.51	n.d.*	7.11	0.2
Izvoare	$9.00 \cdot 10^4$	$4.00 \cdot 10^3$	10.14	n.d.*	5.01	0
Rast	$2.60 \cdot 10^5$	$6.40 \cdot 10^3$	6.41	n.d.*	4.73	0.35
Dragotesti	$2.50 \cdot 10^5$	$2.00 \cdot 10^4$	12.02	n.d.*	4.9	0
Leu	$1.00 \cdot 10^6$	$6.00 \cdot 10^3$	25.5	n.d.*	10.34	0.03
Calafat	$5.50 \cdot 10^6$	$1.00 \cdot 10^3$	5.05	n.d.*	3.76	0
Călăraşi county						
Belciugatele	$2.60 \cdot 10^6$	$8.00 \cdot 10^3$	7.29	n.d.*	11.49	0.15
Lupsanu	$1.10 \cdot 10^5$	$8.00 \cdot 10^3$	6.67	n.d.*	16.72	0.4
Plataresti	$1.70 \cdot 10^5$	$1.20 \cdot 10^4$	298.72	n.d.*	4.39	0
Dor Marunt	$1.40 \cdot 10^5$	$4.00 \cdot 10^3$	5.11	n.d.*	14.69	0.18
St. Voda	$6.40 \cdot 10^5$	$2.10 \cdot 10^4$	9.67	n.d.*	38.52	0.15
Al. Odobescu	$2.70 \cdot 10^5$	$1.90 \cdot 10^4$	6.44	n.d.*	20.85	0.13
Mitreni	$4.70 \cdot 10^5$	$3.50 \cdot 10^4$	9.59	n.d.*	19.32	1.23
Independentei	$8.20 \cdot 10^4$	$1.20 \cdot 10^4$	8.54	n.d.*	15.01	0.2
Vlad Tepes	$3.10 \cdot 10^5$	$7.00 \cdot 10^3$	5.25	n.d.*	22.82	0.03
Fundeni	$4.10 \cdot 10^5$	$8.00 \cdot 10^3$	397.8	0.3709	4.43	0
Sohatu	$7.00 \cdot 10^4$	$4.00 \cdot 10^3$	125.84	n.d.*	1.71	0.02
Galbinasi	$1.90 \cdot 10^5$	$1.80 \cdot 10^4$	147.56	n.d.*	15.19	0
Olt county						
Slatina Z3	$3.20 \cdot 10^5$	$5.00 \cdot 10^4$	52.78	n.d.*	10.73	0.1

Locality	NTG (ufc/g)	YM (ufc/g)	DON (□g/kg)	ZEA (□g/kg)	Impurities (%)	Moldy grains (%)
Studina Z9	$1.10 \cdot 10^6$	$1.40 \cdot 10^4$	0.09	n.d.*	18.74	0.48
Corabia Z10	$7.20 \cdot 10^5$	$1.00 \cdot 10^4$	44.2	n.d.*	15.4	0.07
Samburesti Z1	$4.40 \cdot 10^5$	$9.00 \cdot 10^4$	45.4	n.d.*	7.25	0
Piatra Olt Z5	$5.30 \cdot 10^5$	$8.20 \cdot 10^4$	29.04	n.d.*	10.39	0
Bals Z4	$4.50 \cdot 10^5$	$1.10 \cdot 10^4$	233.46	n.d.*	13.76	0
Valea Mare Z6	$1.90 \cdot 10^5$	$1.00 \cdot 10^3$	15.79	n.d.*	6.05	0
Ianca Z11	$8.70 \cdot 10^5$	$2.10 \cdot 10^4$	18.36	n.d.*	17.88	0.22
Dâmbovița county						
Dobra	$1.60 \cdot 10^5$	$7.00 \cdot 10^3$	27.01	n.d.*	6.71	0
Contesti	$8.00 \cdot 10^4$	$1.00 \cdot 10^2$	686.51	n.d.*	4.99	0
Selbru, Visina	$4.30 \cdot 10^5$	$1.20 \cdot 10^4$	58.55	n.d.*	8.89	0
Dragodana, Matasaru	$1.60 \cdot 10^5$	$7.30 \cdot 10^4$	390.17	n.d.*	4.85	0
Buzău county						
Pogoanele, Scutelnici	$1.30 \cdot 10^6$	$3.60 \cdot 10^4$	n.d.*	n.d.*	10.26	0.25
Vadu Pasii, Sageata	$1.50 \cdot 10^5$	$1.20 \cdot 10^3$	n.d.*	n.d.*	7.46	0.2
Padina, Luciu	$2.50 \cdot 10^5$	$9.40 \cdot 10^3$	n.d.*	n.d.*	16.04	0.3
Puiesti, Boldu	$2.20 \cdot 10^5$	$7.00 \cdot 10^2$	n.d.*	n.d.*	9.42	0.32
Susetu, C.A.Roseti	$5.30 \cdot 10^5$	$5.00 \cdot 10^3$	n.d.*	n.d.*	12.02	0.15
Mihailesti	$1.40 \cdot 10^5$	$1.10 \cdot 10^4$	n.d.*	n.d.*	26.89	0.13
Gl. Silistea	$2.50 \cdot 10^5$	$6.00 \cdot 10^3$	n.d.*	n.d.*	9.32	0.08
P. Calmau	$1.90 \cdot 10^5$	$7.40 \cdot 10^3$	17.98	n.d.*	16.36	0.32
Puiesti, Boldu	$5.10 \cdot 10^5$	$5.50 \cdot 10^3$	31.28	n.d.*	9.81	0.02
Brăila county						
Brăila Z1	$3.40 \cdot 10^5$	$1.30 \cdot 10^4$	64.07	10.101	5.55	0.08
Brăila Z2	$3.10 \cdot 10^6$	$6.70 \cdot 10^4$	n.d.*	n.d.*	12.55	0.72
Brăila Z3	$1.00 \cdot 10^5$	$1.60 \cdot 10^3$	n.d.*	n.d.*	17.26	0.13
Brăila Z4	$3.50 \cdot 10^5$	$3.90 \cdot 10^3$	94.96	0.63224	7.71	0.65
Mehedinți county						
Burila Mare	$1.30 \cdot 10^5$	$1.70 \cdot 10^3$	n.d.*	n.d.*	3.17	0.2
Vanju Mare	$3.10 \cdot 10^6$	$2.50 \cdot 10^3$	n.d.*	n.d.*	9.73	0.05
Garla Mare	$1.40 \cdot 10^5$	$1.80 \cdot 10^4$	n.d.*	n.d.*	6.46	0.07
Teleorman county						
Nasturelu	$1.00 \cdot 10^5$	$3.30 \cdot 10^3$	n.d.*	n.d.*	14.81	0
Smardioasa	$3.50 \cdot 10^5$	$5.20 \cdot 10^4$	n.d.*	n.d.*	28.94	0.23
Turnu Magurele	$1.40 \cdot 10^7$	$4.00 \cdot 10^3$	n.d.*	n.d.*	11.74	0.17
Balaci	$3.50 \cdot 10^5$	$1.70 \cdot 10^4$	22.37	n.d.*	20.84	0
Magura	$4.10 \cdot 10^5$	$7.00 \cdot 10^3$	74.59	n.d.*	15.25	0.1
Zimnicea	$2.40 \cdot 10^6$	$7.90 \cdot 10^5$	n.d.*	n.d.*	12.1	1.06
Traian	$2.20 \cdot 10^6$	$1.10 \cdot 10^3$	n.d.*	n.d.*	24.37	0
Bogdana	$4.60 \cdot 10^5$	$1.70 \cdot 10^3$	185.1	112.657	24.48	0.08
Botoroaga	$1.60 \cdot 10^5$	$5.20 \cdot 10^3$	17.5	n.d.*	15.05	0.28
Ciolanesti	$3.50 \cdot 10^5$	$3.10 \cdot 10^3$	n.d.*	n.d.*	7.39	0
Calmatui	$4.80 \cdot 10^5$	$9.10 \cdot 10^3$	n.d.*	n.d.*	27.17	0
Piatra	$1.10 \cdot 10^5$	$2.00 \cdot 10^3$	n.d.*	n.d.*	11.88	0.07
Mosteni	$7.50 \cdot 10^5$	$5.20 \cdot 10^3$	279.94	191.052	26.5	0.05
Vitanesti	$1.50 \cdot 10^5$	$1.40 \cdot 10^5$	n.d.*	n.d.*	6.04	0

Locality	NTG (ufc/g)	YM (ufc/g)	DON (□g/kg)	ZEA (□g/kg)	Impurities (%)	Moldy grains (%)
Rosiori de Vede	$1.10 \cdot 10^5$	$3.60 \cdot 10^3$	n.d.*	n.d.*	4.87	0.05
Videle	$7.00 \cdot 10^5$	$3.00 \cdot 10^3$	n.d.*	n.d.*	7.7	0

* not detected

In Figure 1 is presented the evolution of DON content in all the wheat samples. As it can be noticed, the highest value for DON is registered in Dâmboviţa (686.51 g/kg), followed by Dolj (554.39 g/kg) and Călăraşi (397.8 g/kg).

In all wheat samples studied, the mycotoxin content was below the maximum levels allowed for unprocessed cereals established by CE no. 1881/2006 regulation; except for Teleorman county where samples from Bogdana and Mosteni locality exceed the limit for ZEA content (100 g/kg), namely 112.657 g/kg, respectively 191.052 g/kg (Fig.2).

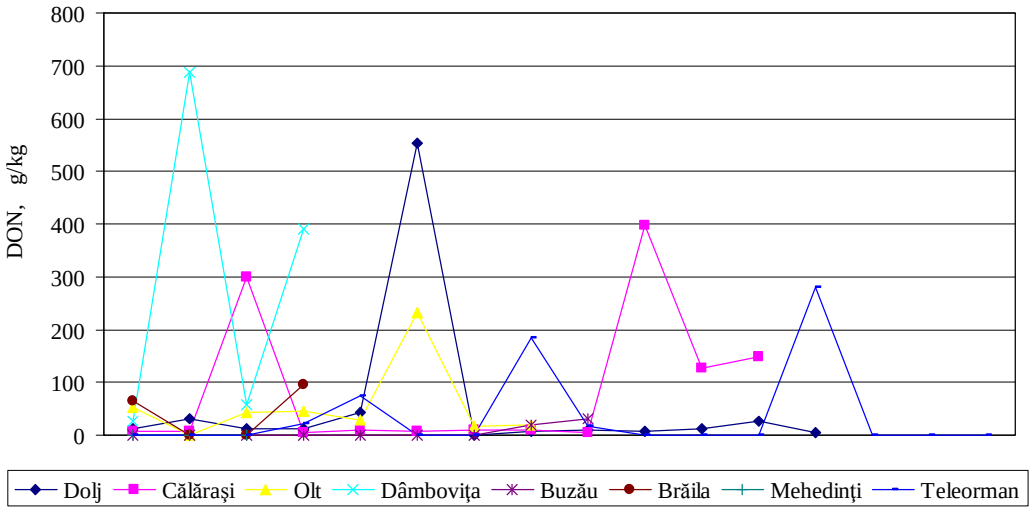

Fig.1. Evolution of DON content in wheat samples from the counties analyzed.

There were samples in which mycotoxin content was not detected: 92.7% of samples analyzed for ZEA, respectively 34.8% for DON content, the values being below the limit of detection of the method used for analysis. So, DON presence is higher than ZEA in wheat samples studied.

In 5 counties, namely, Dolj, Olt, Dâmboviţa, Buzău and Mehedinţi, ZEA content was not detected in all the wheat samples studied. The only values detected for ZEA content in some localities from Călăraşi, Brăila and Teleorman are presented in Fig.2.

Also, in all the samples from Călăraşi, Olt and Dâmboviţa, DON content was detected.

In Fig.3 is shown a comparison between the highest values for DON found in a wheat sample from each of the county analyzed and the corresponding value for ZEA for the same sample.

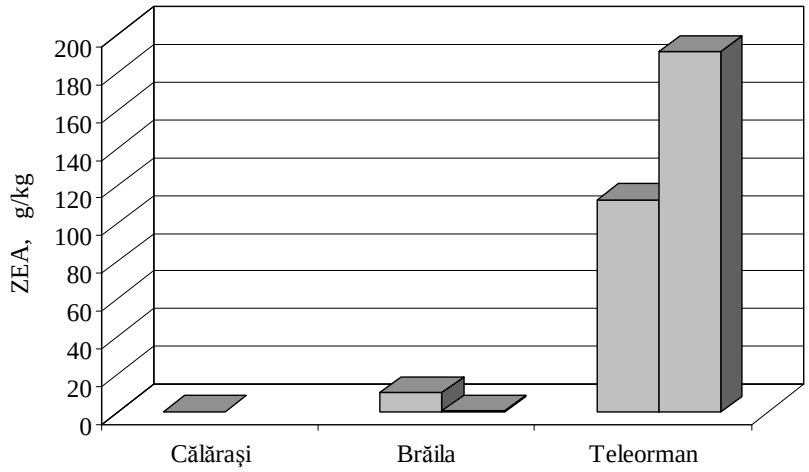

Fig.2. ZEA content in wheat samples analyzed.

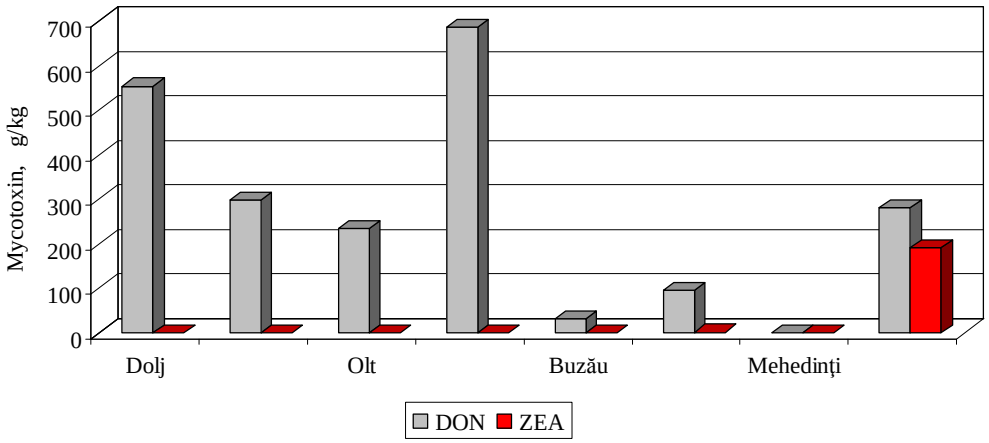

Fig.3. Comparison between DON and ZEA content in the same locality.

4. CONCLUSIONS

In this study, for all wheat samples (*Triticum aestivum*, Dropia variety) studied from 8 different regions situated in southern Romania, the mycotoxin content was below the maximum levels allowed for unprocessed cereals established by European Regulation, except for Teleorman county were 2 samples exceed the limit for ZEA content.

In the same county, Dropia variety had different mycotoxin loads, varying from below the limit of detection, of the method used for analysis, to higher values.

DON presence is higher than ZEA in wheat samples studied. The analyses have been done immediately after harvest; therefore the content of mycotoxins is low. This indicates a good quality of the wheat samples studied in this regard.

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THE POLYOLEFIN PACKAGE INFLUENCE UPON THE QUALITY OF THE BREAD ENRICHED WITH EXOGENOUS BUCKWHEAT ADDING

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Abstract. *The testing of the package for the bread products had as purpose not only the evaluation of the "food contact" quality but also the barrier properties for gases and water vapors. As a bread product we chose the bread enriched with exogenous buckwheat adding. There were tested four types of polyolefin packagings for the global migration of the components in different food simulants and we also led the heavy metals determination using a atomic absorption spectrophotometer. For the permeability tests we used devices for determining the permeability at gases and water vapors. The bread enriched with exogenous buckwheat adding was analyzed from the point of view of the technological quality indicators and the nutritional point of view by analyzing for the later the mineral substances content. The experiments underlined on one hand the benefits of the buckwheat adding in the panification process and proved on the other hand the fact that the packaging prolongues the shelf life ensuring the microbiological quality and the relative constant preservation of the characteristics of the finished product.*

Keywords: ***polyolefin package, buckwheat bread, global migration, witness sample, permeability***

1. INTRODUCTION

The using of the buckwheta adding in bread fabrication was due to the fact that this one is found at the border between food stuff and medicine.Common buckwheat (Fagopyrium esculentum Moench) is recognized as an important functional food in some countries such as China, Japan and Taiwan.[3] Phenolic compounds in buckwheat have been shown to possess antioxidant activity. Four flavonol glycosides including rutin, quartering, kaemferol-3-rutinoside and a trace amount of a flavanol triglyco- side were found in the methanol extract of buckwheat.[4] Compared to most fruits, vegetables and grain crops, buckwheat contains more rutin, which is a quercetin-3-rutinoside with antioxidant, anti-inflammatory and ant carcinogenic effects, and can also reduce the fragility of blood vessels related to hemorrhagic disease and hypertension in humans.[1] In addition, buckwheat was found to be a prebiotic food because it could increase lactic acid bacteria in rat intestine. It is claimed that buckwheat grain extract could be used in the treatment of allergic inflammation.[5] Buckwheat could be incorporated into bread and provide buck- wheat enhanced wheat bread with more functional components and more effective antioxidant properties.[2]
For the prolonging of the preservation time and the ensuring of the product quality keeping is advisable for the products to be packed.[7]

The olefynic materials usually used for the bread products packaging are thin films most of them of polypropylene (PP), polyethylene (PE) or multilayer type.[6] In this study

we analyzed the permeability for gases and water vapors for the bread packagings for the inner-outer and viceversa oxygene transfer and water vapors is linked to the apearance of several alterations of the packed product.

The packing layers permeability is the determinant parameter for evaluating their barrier properties in the mass transfer (Journal of Food Engineering 89, 2008). For to appreciate a material's innocuity in direct contact with the food stuff there was determined the ceding degree for the different compounds of a package in an extraction liquid.

2. MATERIALS AND METHODS

In order to obtain some available experimental data, wheat flour obtaining from FLAMURA wheat variety grinding in Chopin Laboratory Mill was used like control sample. The technological characteristics of flour are shown in Table 1.

Table 1. Analytical parameters of Control flour

Moisture %	Ash %	Wet gluten %	Protein %	Hydration capacity %	Falling Number sec
13.42	0.68	31.1	13,3	62.6	329

Buckwheat flour was provided by local producers.

The analytical flour quality was determined according to the international standard methods (ash content – ICC104/1, wet gluten – ICC105/2, protein content – ICC106/2, hydration capacity with Pharinograph - ICC115/1 and Zeleny index – ICC116/1).

Like raw material I used also compacted fresh yeast (*Saccharomyces cerevisiae)* from S.C. ROMPAK, Pascani, with 32.5% dry matter and 46.54% protein content (N x 6.25).

Dough was prepared using a straight dough method. After baking, the samples were cooling 6-8 hours in controlled atmosphere (UV lamps).

In order to be scoring (after 24 hours), and to be examined from microbiological point of view (after 72 hours), the samples were sliced for packed in plastic bags

Microbiological quality of bread with buckwheat, over 3 days at room temperature was evaluated according with SR EN ISO 4832/2006 quality standard.

The mineral substances were evaluated using EDX-900.

For the bread packing there were used polypropylene bags (PP), high density polyethylene (HDPE), low density polyethylene (LDPE), multilayer foil (PE/ adhesive / PA/ adhesive/ PE) supplied by Sudpack.

The determination of the permeability at gases was led according to the BS EN ISO 2556/2003 standard, using the Dansensor 1 L 100-5000 device. The determination of the permeability at water vapors was led according to the SR EN ISO 15106-1/2005 standard, using the Dansensor 1 L 80-5000 device.

For the components global determination we used as food simulants distilled water, 3% acetic acid and isooctane according to STAS 1186-1/2003. The working method for determining the Pb and Cd migration from the plastic materials is similar to the one in SR ISO 1: 19997, the exception being made by the acetic acid solution that in our case had a

concentration of 3% and the extract was analyzed after the sterilization and concentration by atomic absorption spectrophotometry in oxyacetylene flame of A Analyst 400 type with a background sound correction.

3. RESULTS AND DISCUSSIONS

The analysis of the gases and water vapors permeability of the polyolefin packaging consisted in the determination of the transmission speed of the gas through thin foils under atmospheric pressure in the case of the gases and the determination of the transmission speed of the water vapors for the water vapors permeability.

The testing of the permeability of the polyolefin foils underlined the high water vapors and gases barrier of the multilayer foil followed by the PP and HDPE foils. The lowest barrier properties were presented by the LDPE foil.

The migration test conditions in distilled water and acetic acid were: 10 days at 40◦C, and for the isooctane 24 hours at 40◦C.

Table 2. The permeability analysis results

No.	Sample	Permeability at gases ($cm^3/m^2/day$)		Permeability at vapors ($g/m^2/zi$)
		oxygene (23 ^{0}C), $cm^3/m^2/day$	CO_2 (23^{0}C)	Water vapors (23 ^{0}C)
1	PP foil bag	1900	8900	4,21
2	HDPE foil bag	2123	96000	5,7
3	LDPE foil bag	6398	36000	25,1
4	PE multilayer foil bag and welding layer Surlyn ionomer and EVOH barrier	16	2,7	1

After the evaluation of the global migration level we observed that all the migration values are under the limit of 60 ppm (10mg/dm^2), imposed by the laws.

After the organoleptic examination and the realization of the extractions in the established conditions of the working field the package samples studied destined for food contact didn't present visible changes of the colour and appearance of the surface in comparison with the witness samples. At the same time the food simulants didn't present colour, transparency (opalescence), smell or taste (where possible) changes in comparison with the witness samples.

The evaluation of the AAS analysis of the heavy metals migration took into account the ORD. Nr.975/1998, that specified the limits for: Pb:0,1ppm and Cd absent. We observed that most of the values were under the LD detection limit of the device, with two exceptions in the case of Pb (the PP and LDPE foils).

The analysis of the technological and physico-chemical quality of the bread packed in polyolefin package

For evaluating the quality of the bread with buckwheat adding we made two samples one with high gluten wheat flour and one with 10% Buckwheat flour adding.

Table 3. The results of the global migration tests in the simulants A, B and D and the heavy metals migration

Name	Extraction environment	Extraction conditions /extraction ratio	Organoleptic modifications		Global migration ppm	Metals ceding ppm	
			Sample	Extract		Pb	Cd
1. PP foil bag	Distilled water	10 days, 40 °C/1:2	Unmodified	Unmodified	5,25	-	-
	Acetic acid sol. 3%	10 days, 40 °C/1:2	Unmodified	Unmodified	5,5	0,017	<LD
	Isooctane	48h, t.c./1:2	Unmodified	Unmodified	7,25	-	-
2. HDPE foil bag	Distilled water	10 days, 40 °C/1:2	Unmodified	Unmodified	6.0	-	-
	Acetic acid sol. 3%	10 days, 40 °C/1:2	Unmodified	Unmodified	6.5	-	<LD
	Isooctane	48h, t.c./1:2	Unmodified	Unmodified	8.0	-	-
3. LDPE foil bag	Distilled water	10 days, 40 °C/1:2	Unmodified	Unmodified	6.5	-	-
	Acetic acid sol. 3%	10 days, 40 °C/1:2	Unmodified	Unmodified	7.5	0.035	<LD
	Isooctane	48h, t.c./1:2	Unmodified	Unmodified	8.75	-	-
4. PE multilayer foil bag and welding layer Surlyn ionomer and EVOH barrier	Distilled water	10 days, 40 °C/1:2	Unmodified	Unmodified	6.0	-	-
	Acetic acid sol. 3%	10 days, 40 °C/1:2	Unmodified	Unmodified	6.5	<LD	<LD
	Isooctane	48h, t.c./1:2	Unmodified	Unmodified	6.5	-	-

The recipe used for making breads is shown in table 4.

We analyzed the bread in the fresh state and after 4 days of foil package.

The results registered are presented in table 5.

From the mineral substances content point of view (table 6) we observed an improvement in the composition at the buckwheat adding sample.

For we didn't observe any major modifications regarding the quality of the finished products (the quality indicators) and taking into account the functional food qualities shown we choose for continuing our researches the buckwheat bread. We made the following samples:

Witness sample/- bread with 3% buckwheat at 3 hours after baking

P1 Unpacked bread with 10% buckwheat

P2- Bread packed in PP foil with 10% buckwheat

P3- Bread packed in HDPE foil with 10% buckwheat

P4- Bread packed in LDPE foil with 10% buckwheat
P5- Bread packed in multilayer foil with 10% buckwheat

Table 4. Recipe for bread with buckwheat flour

Ingredients and technological regime	UM	Quantities
Raw materials and ingredients		
Wheat flour	grams	500
Buckwheat flour	grams	
Fresh yeast	grams	8
Salt	grams	7
Water	cm^3	270 to 300
Technological parameters		
Kneading time	min	10-15
Proffer time	min	20-25
Temperature of blanks	^0C	30-32
Baking time	min	30-35
Baking temperature	min	240-260

Table 5. The physico-chemical analysis of the samples

Physico-chemical indicator	Witness sample (bread without buckwheat adding)	Sample with 10% buckwheat adding
Height/ dimenssion ratio	0.62	0.64
Volume, cm^3/100g	300	320
Porosity, [%]	35	30
Elasticity, [%]	78	63,65
Acidity, acidity grades/100 g	0.6	1,4
Humidity, [%]	69.46	78,78

Table 6. The mineral composition of the bread samples before packing

Mineral substances	Cl	K	P	Ca	S	Fe	Cu	Mn	Zn	Br	Rb
Witness sample	61,09	24,30	9,519	3,067	1,555	0,193	0,063	0,086	0,136	0,035	0,019
Bread with 10% buckwheat	61,26	27,71	11,602	5,161	1,555	3,173	0,063	0,086	0,136	0,035	0,019

Analyzing the obtained data we saw a relative constant maintenance of the physico-chemical indicators excepting the humidity which is obviously higher at the unpacked bread in comparison with the packed one. The highest humidity level was at the P5 multilayer foil package for the other samples being observed a reduction of the values in comparison with the witness samples. The physico-chemical and technological indicators that were the closest to the values obtained before tha packing were determined for the

bread packed in the multilayer foil bag. The packaging didn't influence the mineral substances content.

Table 7. The physico-chemical analysis of the packed bread samples

Physico-chemical indicator	Witness sample after 3 hours of baking	The sample after 4 days				
		P1 Unpacked bread with 10% buckwheat	P2 Bread packed in PP foil with 10% buckwheat	P3 Bread packed in HDPE foil with 10% buckwheat	P4 Bread packed in LDPE foil with 10% buckwheat	P5 Bread packed in multilayer foil with 10% buckwheat
Height/ dimenssion ratio	0,64	0,64	0,60	0,58	0,41	0,63
Volume, cm^3/100g	320	310	315	315	315	315
Porosity, [%]	30	30	25	24	21	28
Elasticity, [%]	63,65	61,65	56,23	56,01	53,13	61,98
Acidity, acidity grades/100 g	1,4	1,4	1,4	1,4	1,4	1,4
Humidity, [%]	78,78	81,7	73,77	73,12	68.21	76.64

The microbiological analysis of the sample

Because the final contamination depends of the initial level of microorganisms, it was evaluate the evolution of microbiota starting by 3 hours after breads backing till the fourth day of storage. A qualitative microbiological determination was led to each sample.

Initially the contamination level for all five samples subjected to analysis was null. The packed bread samples didn't present in the first three days any important modifications from the microbiological point of view.

In the fourth day we observed modifications of the surface at P5 (the bread packed in multilayer foil) and also at the unpacked bread duet o the appearance of mold at the surface (fig. 1). After the sampling and microscopic examination we determined that the mold belongs to the Aspergillus (fig. 2) and Mucor types (fig. 3).

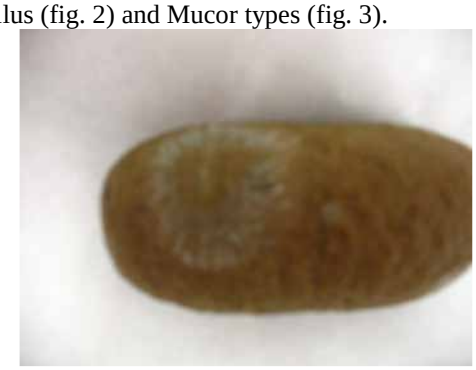

Fig 1 Mold at the buckwheat bread surface

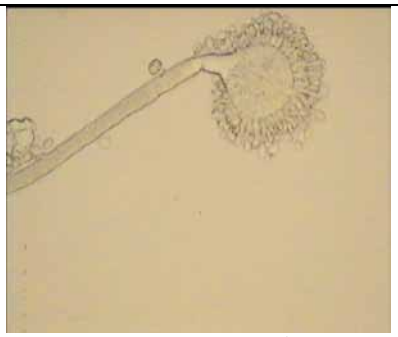

Fig.2. Aspergillius flavus

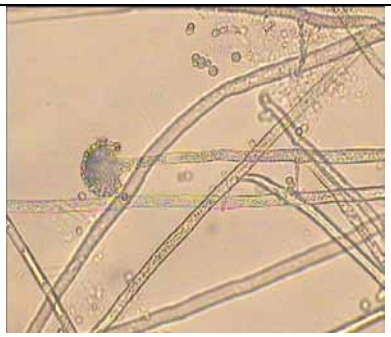

Fig.3. Mucor mucedo

The Aspergillius flavus colonies are light- green coloured having a fine granular appearance. The aspergilla ends are globular at the microscope; the sustaining filaments are strong and well developed with biseries serigma.

The Mucor mucedo colonies developed on the surface of the bread are soft of white-greysh colour. At the microscope the hyphae are well branched and linked in a thick network.

This mold development is determined by the high water content of the two samples (81,7% humidity at the witness sample and 76.64% at the P5 sample) in comparison with the other samples.

CONCLUSIONS

Substituting 10% of wheat flour in the bread formula with buckwheat flour would not interfere with bread specific volume and score.

The increasing of the mineral substances (K,P,Ca,Fe) in the bread with buckwheat adding is benefic for the body for the calcium ions balance the cellular membranes the potassium adjusts the hydro-salin and intracellular metabolism as well as the osmotic pressure and the iron enters the myoglobin structure transporting the oxygene and the phosphorus is of great importance for the stability of the human bone structure.

The studied foils present a average permeability at gases, and a good permeability at water vapors and smells. The best water vapors and gases permeability is attributed to the multilayer foil followed by the PP and HDPE foils. In the case of evaluating the global migration level we observed that all the results are under the limit imposed by the law. The heavy metals migration analysis showed a slight increasement of the Pb in the case of PP and LDPE foils without making them unproper for using.

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SECTION VI: MISCELLANEOUS

RESEARCH ABOUT INFLUENCE OF DENSITY AND FERTILISATION ON PROTEIN AND STARCH CONTENT IN SOME GENOTYPES OF BARLEY, VARIETY DISTICHUM

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Abstract. *This paper presents results on the evolution of protein and starch content of barley varieties, variety distichum, Annabelle, Thuringia, and Cristalia combined under differential fertilization (N0P0, N40P40, N80P80, N120P120) and different seeding densities (400 bg/m^2, 600 bg/m^2).*
The average results obtained for the factors studied in the three experimental years (2007-2008, 2008-2009 and 2009-2010), in the Vadeni, Braila county, shows that total protein varied between 9.85% and 13.2% and starch content between 61.86% and 64.1%.

Keywords: barley, density, fertilization, protein content, starch content

1. INTRODUCTION

The main components of barley grain to be taken into account in determining the destination for the manufacture of malt and beer are protein and starch content. The content of protein substances, expressed as a percentage of dry matter, is a characteristic of the variety, but at the same time is strongly influenced by climatic conditions and the way culture, vary widely within one and the same variety from year to year and from one place to another. A grain of starch content determined by the highest amount of extract and varies widely between 57-65% of dry matter. To the extent that the value of this indicator is higher, shows better quality barley for malting. [1], [2], [3].

This indicator is in an inverse ratio to the protein content. Research conducted in different soil conditions have shown that along with genetic factors, climatic factors contribute to changes in technology broadly chemical composition and quality of crops (*Borlan Z., et al., 1994*)

Fertilizers influence the quality of barley, therefore they should be applied according to the purpose for which the culture (feed or malt for beer).

2. MATERIALS AND METHODS

The investigations were made during the agricultural years 2007-2008, 2008-2009, 2009-2010, in the Vadeni, Braila County, on a limestone soil type aluvial soil. The biological material used was the experience of three spring barley varieties: Annabell, Thuringia, and Cristalia.

Experience trifactorial AxBxC type was placed by the method of parcels subdivided in three repetitions. Factors studied were:

Factor A - fertilization, with graduations:

a_1 - N0P0

a_2 - N40P40

a_3 - N80P80

a_4 - N120P120

Factor B - variety, with graduations:

b_1 - Annabell

b_2 - Thuringia

b_3 - Cristalia

Factor C - sowing density with graduations:

c_1 - 400 bg/m^2

c_2 - 600 bg/m^2

The objectives of this work is the determination of protein and starch accumulated by the three spring barley varieties under the influence of the interaction level of fertilization and planting density in the three years of research. Determination of protein and starch was done by a simple and modern, using the device OmegAnalyzer G.

Statistical processing of results was done by the method of analysis of variance, using MS Office Excel - ANOVA test. As a witness to use the average of the variants.

3. RESULTS AND DISCUSSIONS

The results were summarized and recorded in the tables below.

To obtain beer content of beans barley protein substances should not be over 12% of dry matter above this limit as barley malts malţifică difficult and results in a lower yield in the extract.

With regard to crude protein content of spring barley, the variety Annabell, in 2008, it ranged from 9,5% in variant N0P0x600bg/m^2 reaching 12,0% in version N120P120x400bg/m^2 values content protein, the variety ranges from 10,1% in Thuringia N0P0x600bg/m^2 and 12,5% in version N120P120x400bg/m^2.

Cristalia variety recorded values of crude protein content from 10% in version N0P0x600 bg/m^2 and 12,2% in version N120P120x600 bg/m^2 .

In 2009 the protein content ranged from 10,2% in variety Annabell (variant N0P0x600bg/m^2) and 12,2% (variant N120P120x400bg/m^2), between 10,5% (variant N0P0x600bg/m^2) and 12,8% (variant N120P120x400bg/m^2), the variety Thuringia and the crude protein content variety Cristalia ranged from 10% (variant N0P0x600bg/m^2) and 12,6% (variant N120P120x400bg/m^2).

Crude protein content recorded in 2010 by the three varieties analyzed ranged from 9,7% (variant N0P0x600bg/m^2) and 12,0% (variant N120P120x400bg/m^2) between 10,3% (variant N0P0x600bg/m^2) and 12,6% (range N120P120x400bg/m^2), and between 10% (range N0P0x600bg/m^2 and version N0P0x400bg/m^2) and 12,3% (variant N120P120x600 bg/m^2).

Mean highest protein content was recorded version ThuringiaxN120P120x400bg/ m^2 and the lower version CristaliaxN40P40X600 bg/$m^{2.}$ (fig.1)

The content of starch grains, influence the highest amount of extract and high values of this index leads to a good malting quality. Thus, varieties with a starch content of at least 57-60% will be very good for malting.

Table 1. Summary of results regarding the influence of experimental factors on the protein content in barley grains

Variant	Protein content (% in dry substance)							
	2008		2009		2010		Average of years	
	%	difference	%	difference	%	difference	%	difference
a1b1c1	11.8	0.94***	12.1	1.24***	11.9	1.04***	11.9	1.04***
a1b1c2	12.0	1.14***	12.2	1.34***	12.0	1.14***	12.1	1.24***
a1b2c1	12.4	1.54***	12.8	1.19***	12.4	1.54***	12.53	1.67***
a1b2c2	12.5	1.64***	12.7	2.24***	12.6	1.74***	12.6	1.74***
a1b3c1	12.2	1.34***	12.4	1.54***	12.3	1.44***	12.3	1.44***
a1b3c2	12.1	1.24***	12.6	1.74***	12.1	1.44***	12.26	1.40***
a2b1c1	10.5	-0.36o	11.2	0.34	10.8	-0.06	10.83	-0.03
a2b1c2	10.8	-0.06	11.1	0.24	10.6	-0.26	10.83	-0.03
a2b2c1	11.0	0.14	11.3	0.44*	11.2	0.36*	11.17	0.31
a2b2c2	11.2	0.34	11.4	0.54**	11.3	0.44*	11.3	0.44*
a2b3c1	10.8	-0.06	11.0	0.14	10.7	-0.16	10.83	-0.03
a2b3c2	10.9	0.04	11.1	0.24	10.9	0.04	10.96	0.1
a3b1c1	10.0	-0.86ooo	10.5	-0.36o	10.6	-0.26	10.37	-0.49oo
a3b1c2	10.2	-0.66ooo	10.4	-0.46oo	10.5	-0.36o	10.37	-0.49oo
a3b2c1	10.8	-0.06	11.0	-0.14	11.0	0.14	10.93	0.07
a3b2c2	10.9	0.04	11.1	0.24	10.8	-0.06	10.93	0.07
a3b3c1	10.5	-0.33	10.7	-0.16	10.6	-0.26	10.6	-0.26
a3b3c2	10.6	-0.26	10.6	-0.26	10.5	-0.36o	10.53	-0.33
a4b1c1	9.5	-1.36 o	10.2	-0.66ooo	9.7	-1.16o	9.8	-1.06ooo
a4b1c2	9.8	-1.06ooo	10.3	-0.46oo	9.9	-0.96o	10.1	-0.76ooo
a4b2c1	10.1	-0.76oo	10.5	-0.36o	10.3	-0.56oo	10.3	-0.56oo
a4b2c2	10.3	-0.56oo	10.7	-0.16	10.4	-0.46oo	10.46	-0.4o
a4b3c1	10.0	-0.86ooo	10.0	-0.86ooo	10.0	-0.86ooo	10.0	-0.86ooo
a4b3c2	10.1	-0.76ooo	10.2	-0.76ooo	10.0	-0.86ooo	10.1	-0.76ooo
Control	Average						**10.86**	-

L 5% = 0,3417% DL 1% = 0,457% DL 0,1% = 0,598%

In 2008 the starch content ranged from 60,0% in variety Annabell (variant N0P0x600bg/m^2) and 64,1% (variant N80P80x600bg/m^2), between 56,9% (variant N0P0x400bg/m^2) and 63,0% (variant N80P80x400bg/m^2), the variety Thuringia and the starch content variety Cristalia ranged from 59,0% (variant N0P0x400bg/m^2) and 63,7% (variant N80P80x400bg/m^2) (fig.2).

In 2009 the starch content ranged from 56,8% in variety Annabell (variant N0P0x600bg/m^2) and 60,7% (variant N80P80x400bg/m^2), between 55,0% (variant N0P0x400bg/m^2) and 60,7% (variant N80P80x400bg/m^2), the variety Thuringia and the starch content variety Cristalia ranged from 56,3% (variant N0P0x600bg/m^2) and 59,4% (variant N80P80x400bg/m^2).

The starch content recorded in 2010 by the three varieties analyzed ranged from 56,6% in variety Annabell (variant N0P0x400bg/m^2) and 63,2% (variant N80P80x600 bg/m^2), between 56,2% (variant N0P0x600bg/m^2) and 60,1% (range N80P80x400bg/ m^2), the variety Thuringia and the starch content variety Cristalia ranged from 57,5% (variant N0P0x600bg/m^2) and 62,8% (variant N80P80x400bg/m^2).

Table 2. Summary of results regarding the influence of experimental factors on the starch content of barley grains

Variant	Starch content (%)							
	2008		2009		2010		Years average	
	%	difference	%	difference	%	difference	%	difference
a1b1c1	60.7	1.82	57.8	-1.08	58.8	-0.08	59.1	0.22
a1b1c2	60.2	1.32	58.2	-0.68	58.6	-0.28	59.00	0.12
a1b2c1	58.8	-0.08	55.0	-3.88^{oo}	57.9	-0.98	57.23	-1.65
a1b2c2	57.0	-1.88	55.2	-3.68^{oo}	57.0	-1.88	56.40	-2.48
a1b3c1	59.1	0.22	56.9	-1.98	58.9	0.02	58.30	-0.58
a1b3c2	58.8	-0.08	56.6	-1.98	60.0	1.12	58.46	-0.42
a2b1c1	64.1	5.22***	60.1	1.22	63.2	4.32**	62.46	3.58**
a2b1c2	64.0	5.12***	60.7	1.82	62.9	4.02**	62.53	3.65**
a2b2c1	62.7	3.82**	58.2	-0.68	60.0	1.12	60.30	1.42
a2b2c2	63.0	4.12**	57.9	-0.98	60.1	1.22	60.33	1.45
a2b3c1	63.4	4.52**	58.8	-0.08	62.3	3.42**	61.50	2.62
a2b3c2	63.7	4.82***	59.4	0.52	62.8	3.92**	61.96	3.08*
a3b1c1	61.3	2.42	58.7	-0.18	59.7	0.82	59.90	1.02
a3b1c2	60.8	1.92	57.9	-0.98	59.3	0.42	59.33	0.45
a3b2c1	57.9	-0.98	55.9	-2.98^{o}	57.0	-1.88	56.93	-1.95
a3b2c2	58.2	-0.68	55.6	-3.28^{o}	56.8	-2.08	56.86	-2.02
a3b3c1	60.1	1.22	58.2	-0.68	58.9	0.02	59.06	0.18
a3b3c2	60.7	1.82	57.7	-1.18	58.2	-0.68	58.86	-0.02
a4b1c1	60.0	1.12	56.8	-2.08	58.9	0.02	58.56	-0.32
a4b1c2	60.3	1.42	57.9	-0.98	56.6	-2.28	58.26	-0.62
a4b2c1	57.3	-1.58	55.3	-3.58^{oo}	56.2	-2.68	56.26	-2.62
a4b2c2	56.9	-1.98	55.0	-3.88^{oo}	56.5	-2.38	56.13	-2.75^{o}
a4b3c1	59.2	0.32	56.3	-2.58	57.5	-1.38	57.66	-1.22
a4b3c2	59.0	0.12	56.6	-2.28	58.0	-0.88	57.86	-1.02
Control	Average						**58.88**	

DL 5% =2,67% DL 1% =3,57% DL 0,1% =4,68

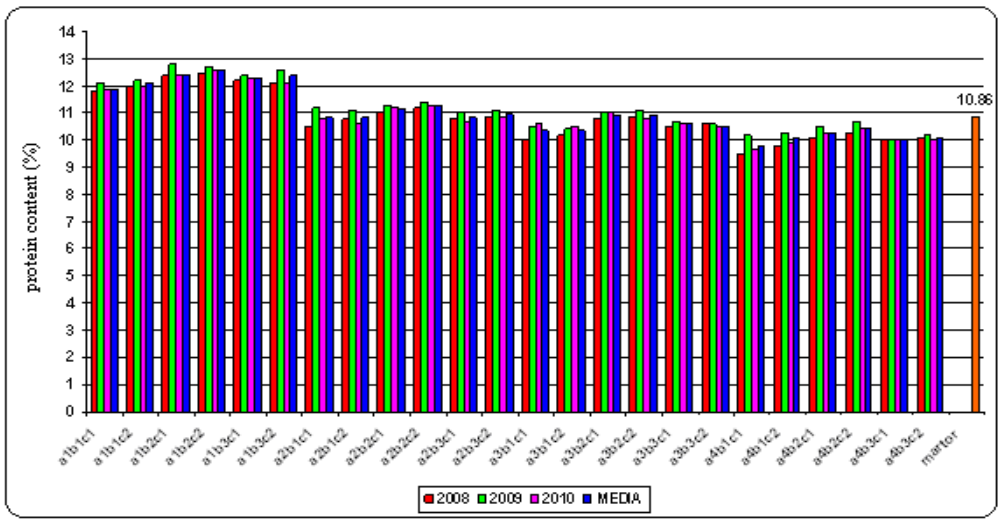

Fig. 1. Crude protein content in every variant of experience

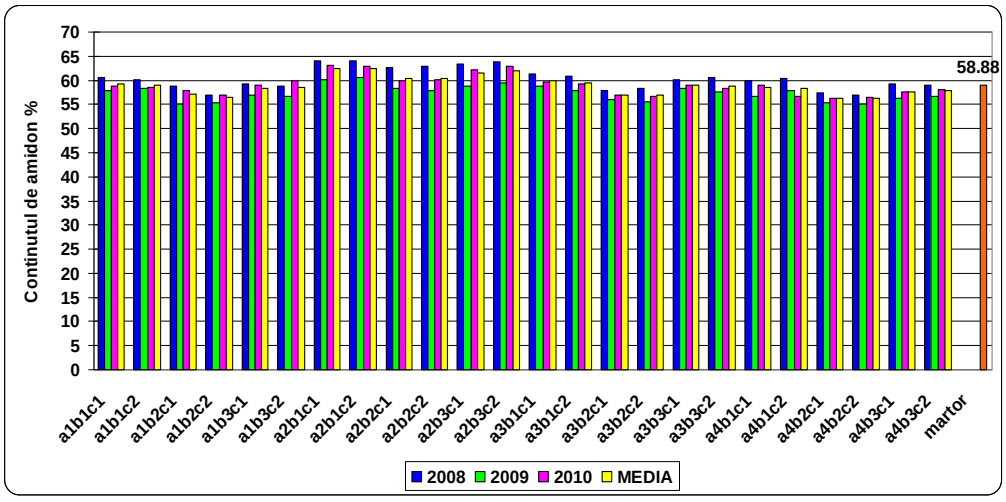

Fig. 2. Starch content in every variant of experience

4. CONCLUSIONS

Interaction of three factors of experience shows that N120P120 doses, regardless of seeding density are favorable for barley for brewing, resulting in a protein content that exceeds 11.5%.

The smallest protein content in grains was recorded at the variant

N0P0xAnnabellx600bg/m^2 with 9,8% and the biggest in variant N120P120xThuringiax400 bg/m^2 with 12,6%.

The smallest starch content in grains was recorded at the variant N0P0xThuringiax400bg/m^2 with 56,13% and the biggest in variant N120P120xAnnabellx400 bg/m^2 with 62,53%.

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STUDY OF CORRELATIONS BETWEEN MAIN QUALITY INDICES OF BARLEY AND TECHNOLOGICAL PARAMETERS

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Abstract. *The paper aims to establish the interdependence between the main quality indices of barley (TCW, MH, assortment, protein content, starch content, range) and main technological parameters of brewing (malt yield, Kolbach index and Hartong index, Extract). It was determinate the linear correlation coefficients between pairs of values in some genotypes of barley (Annabell, Thuringia, Tunika, Boreal) grown in Braila Plain, in three experimental years (2007 - 2010). Experience highlights the importance of quality indicators to obtain quality malt for brewing.*

Keywords: barley, density, fertilization, protein content, starch content

1. INTRODUCTION

Barley is the grain used since antiquity to obtain malt and beer, both because of wide spread culture and characteristics of grain during germination. The beer industry, making a product with superior taste characteristics and corresponding colloidal stability depends primarily on the quality of hexastichym or distichum barley used as raw material.

Barley appreciation for malt production is necessary, as grain quality largely determines the quality of malt and beer, and manufacturing yields. This is done both by appearance and by physical and chemical properties.

Quality barley for brewing has a more complex character, due to the large number of indices that define and having a specific heredity and the fact that they occur in different phases of the process of obtaining malt.

Quality requirements for malting of barley are quite strict and are directly related to processing efficiency and product quality achieved in malt and beer industry. Many of the necessary quality characteristics of barley for beer, are under the control of the manufacturer, others are caused by weather conditions during vegetation and harvesting.

2. MATERIALS AND METHODS

The investigations were made during the agricultural years 2007-2008, 2008-2009, 2009-2010, in the Vadeni, Braila County, on an alluvial soil. The biological material used in experience was three spring barley varieties: Annabell, Thuringia, Tunika, and a variety of winter barley Boreal.

Statistical interpretation of results from laboratory tests were performed using MS Excel - correlation test.

3. RESULTS AND DISCUSSIONS

The results were summarized and recorded in the tables below.

Table 1. Average values of main quality indices

Variety	Quality indices							
	TGM (g)	HM (Kg)	Assortiment (%)	Protein content (%)	Starch content (%)	Kolbach index	Hartog index 45°C	Extract %
Annabell	44,7	65,8	90,1	9,7	64,4	38,8	37,4	78,6
Thuringia	45,1	61,3	92,5	10,8	63,3	45,3	40,5	79,0
Tunika	45,3	65,7	91,2	10,6	65,8	40,7	38,2	79,4
Boreal	44,5	58,9	61,0	11,0	63,0	37,6	36,9	77,8
Average	44,9	62,9	83,7	10,5	64,1	40,6	38,3	78,7

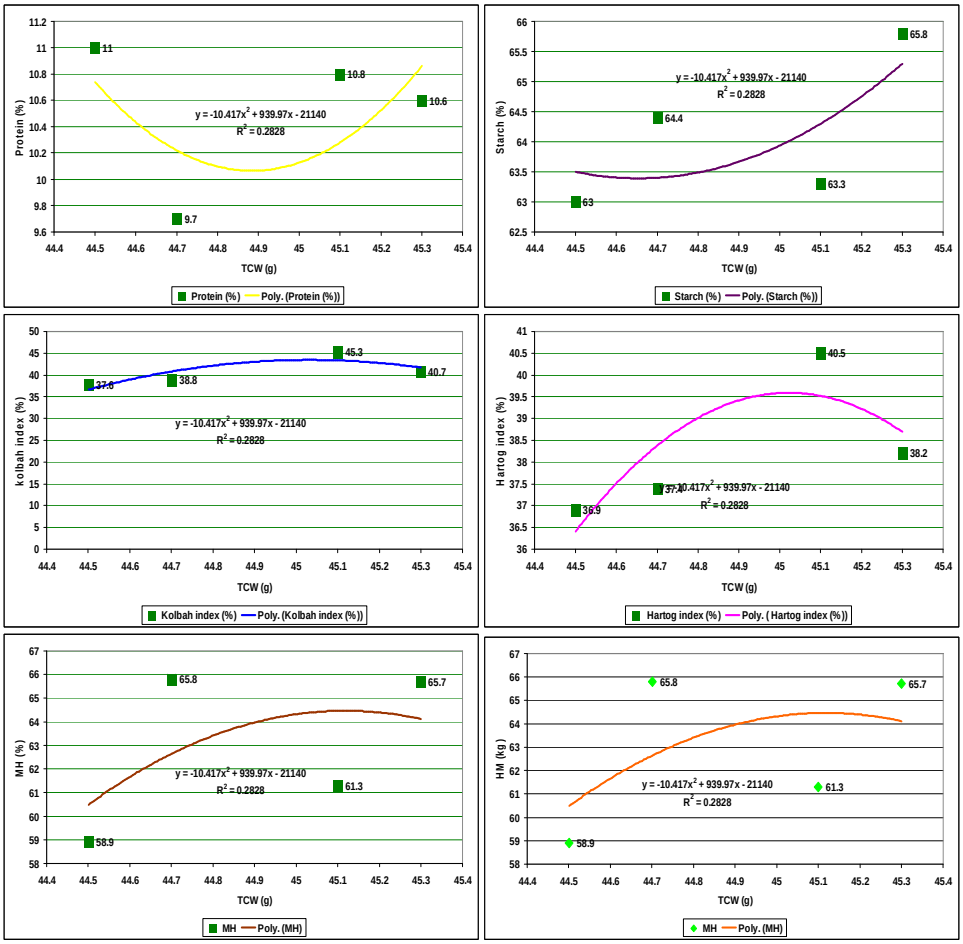
Table 2. Summary of correlations between the main indices of quality of barley grains and main technological parameters

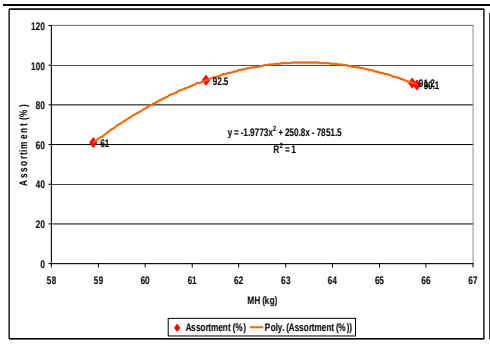
	TCW	HM	Assortment	Protein	Starch	Kolbach	Hartong
HM	0,488 *						
Assortment	0,756 **	0,750 **					
Protein	0,095	-0,771 oo	-0,501 oo				
Starch	0,648 **	0,872 **	0,565 **	-0,395 o			
Kolbach	0,685 **	-0,013	0,641 **	0,233	-0,064		
Hartong	0,653 **	-0,050	0,616 **	0,246	-0,108	0,999 **	
Yield Extract	0,962 **	0,686 **	0,891 **	-0,179	0,735 **	0,629 **	0,594 **

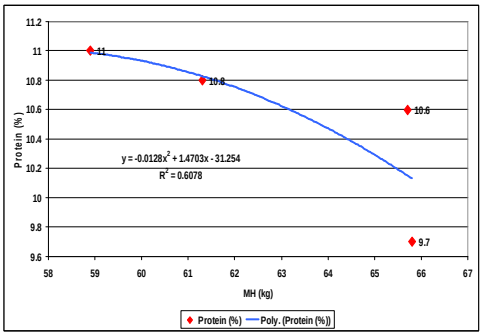
Studying correlations between indices as determined from laboratory tests were found several important correlations. TCW (thousand corn weight) have favorably influence all the indicators analyzed, correlating positive significant and very significant with Assortiment, Kolbach index, Hartong index, starch content of grains and extract.

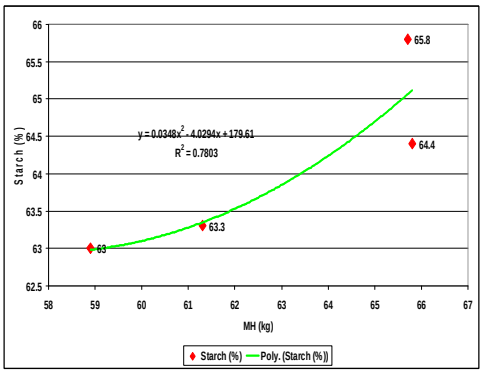
It is remarkable that the coefficient of correlation between the TCW and grain starch content (0,648) was much higher negative correlation coefficient between the content of starch grains and their protein content (-0,395).

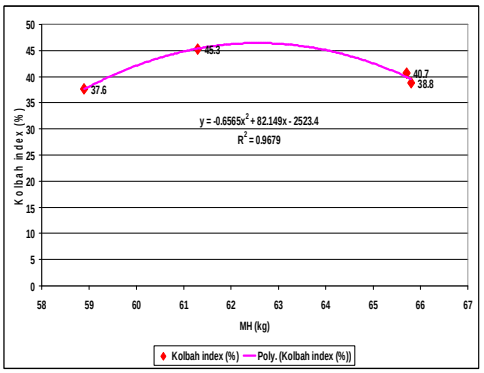
Protein content in grains had a tendency to negative influence other elements analyzed. It was significantly distinctly negatively correlated with HM (-0,771), Assortment (-0,501) and significantly starch content of grains (-0,395).

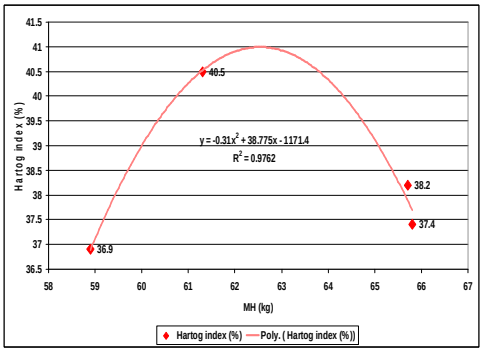


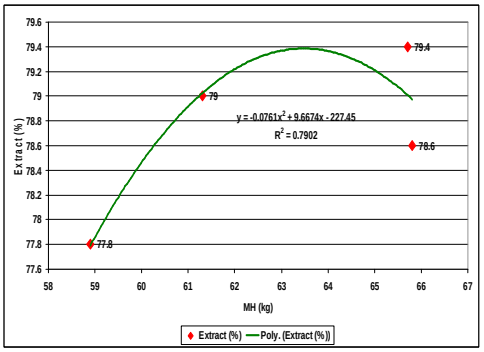

Assortiment (%)
MH (kg)
y = -1.9773x² + 250.8x - 7851.5
R² = 1
Assortment (%)
Poly. (Assortment (%))

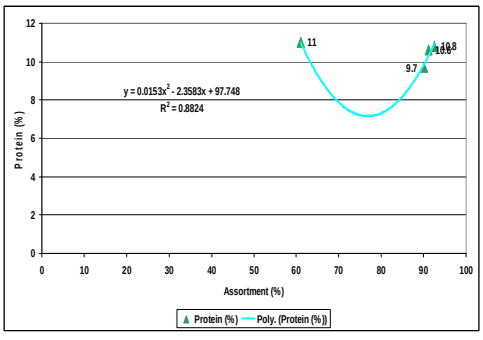

Protein (%)
MH (kg)
y = -0.0128x² + 1.4703x - 31.254
R² = 0.6078
Protein (%)
Poly. (Protein (%))

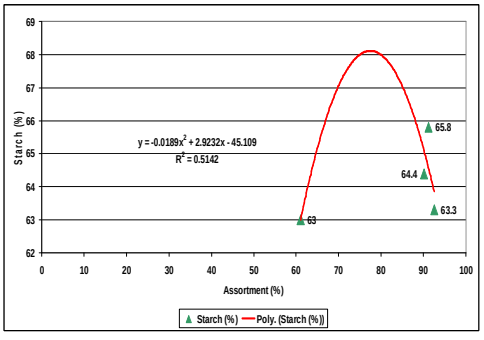

Starch (%)
MH (kg)
y = 0.0348x² - 4.0294x + 179.61
R² = 0.7803
Starch (%)
Poly. (Starch (%))

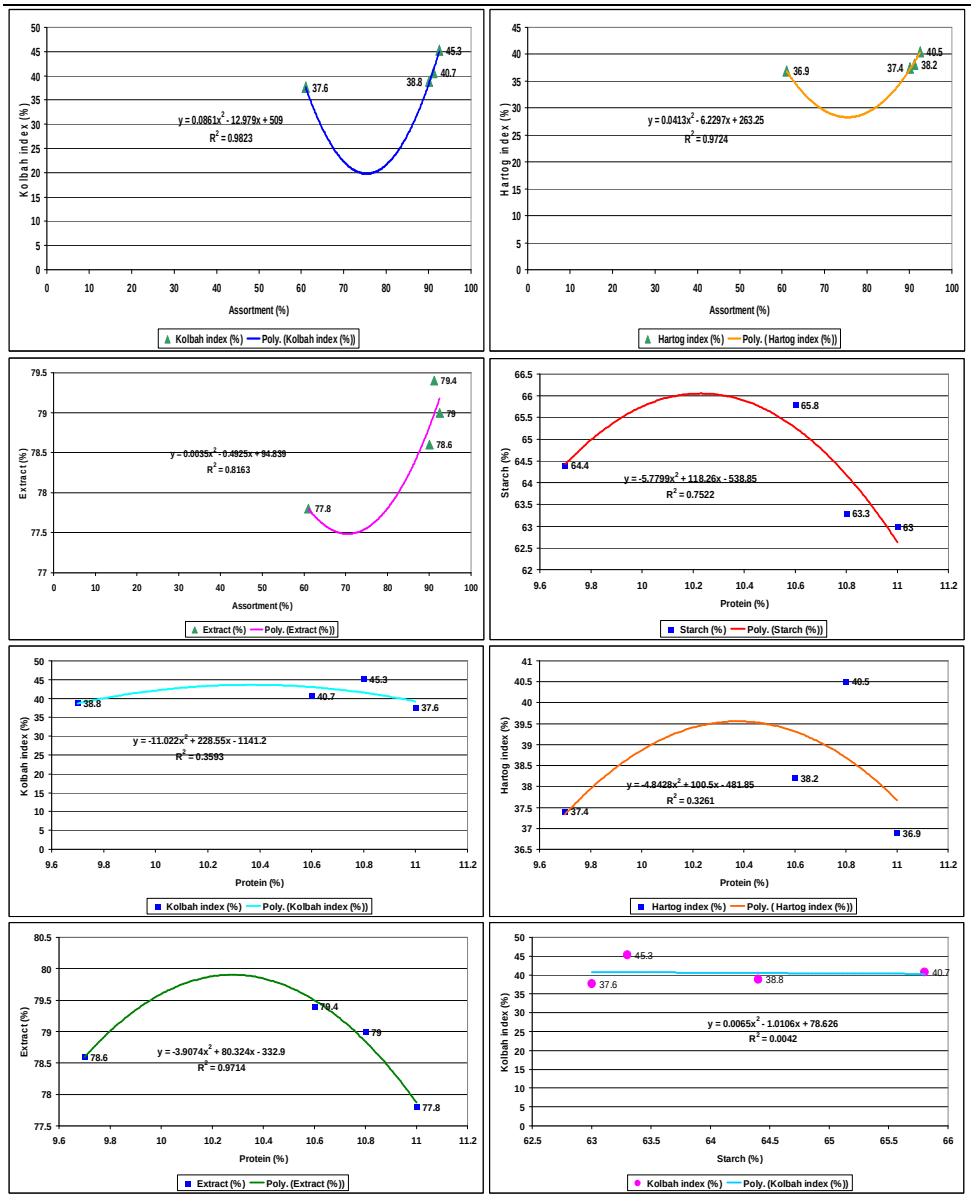

Kolbah index (%)
MH (kg)
y = -0.6565x² + 82.149x - 2523.4
R² = 0.9679
Kolbah index (%)
Poly. (Kolbah index (%))

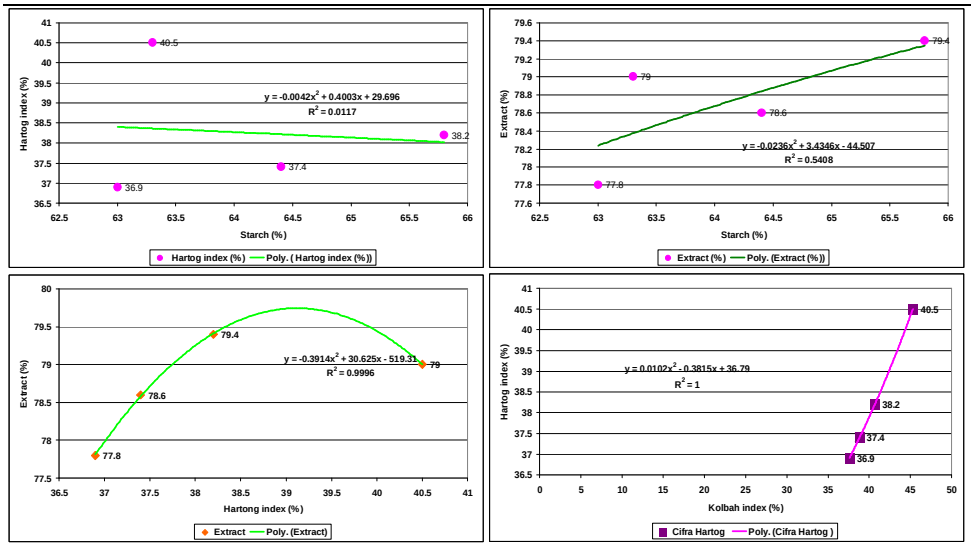

Hartog index (%)
MH (kg)
y = -0.31x² + 38.775x - 1171.4
R² = 0.9762
Hartog index (%)
Poly. (Hartog index (%))


Extract (%)
MH (kg)
y = -0.0761x² + 9.6674x - 227.45
R² = 0.7902
Extract (%)
Poly. (Extract (%))


Protein (%)
Assortment (%)
y = 0.0153x² - 2.3583x + 97.748
R² = 0.8824
Protein (%)
Poly. (Protein (%))


Starch (%)
Assortment (%)
y = -0.0189x² + 2.9232x - 45.109
R² = 0.5142
Starch (%)
Poly. (Starch (%))


Kolbah index (%)
y = 0.0861x² - 12.979x + 509
R² = 0.9823
37.6
38.8
45.3
40.7
Assortment (%)
Kolbah index (%)
Poly. (Kolbah index (%))
Hartog index (%)
y = 0.0413x² - 6.2297x + 263.25
R² = 0.9724
36.9
37.4
40.5
38.2
Assortment (%)
Hartog index (%)
Poly. (Hartog index (%))
Extract (%)
y = 0.0035x² - 0.4925x + 94.839
R² = 0.8163
77.8
79.4
79
78.6
Assortment (%)
Extract (%)
Poly. (Extract (%))
Starch (%)
y = -5.7799x² + 118.26x - 538.85
R² = 0.7522
64.4
65.8
63.3
63
Protein (%)
Starch (%)
Poly. (Starch (%))
Kolbah index (%)
y = -11.022x² + 228.55x - 1141.2
R² = 0.3593
38.8
40.7
45.3
37.6
Protein (%)
Kolbah index (%)
Poly. (Kolbah index (%))
Hartog index (%)
y = -4.8428x² + 100.5x - 481.85
R² = 0.3261
37.4
40.5
38.2
36.9
Protein (%)
Hartog index (%)
Poly. (Hartog index (%))
Extract (%)
y = -3.9074x² + 80.324x - 332.9
R² = 0.9714
78.6
79.4
79
77.8
Protein (%)
Extract (%)
Poly. (Extract (%))
Kolbah index (%)
y = 0.0065x² - 1.0106x + 78.626
R² = 0.0042
45.3
37.6
38.8
40.7
Starch (%)
Kolbah index (%)
Poly. (Kolbah index (%))



Studying correlations between determined quality indices from laboratory tests were found several important connections. Between hectoliter mass and protein content in grains it was observed a negative correlation with a distinct significant correlation coefficient of - 0.771 and significant positive correlations were obtained separately with the assortment (0.750), starch content (0.872), yield of extract (0.686).

Significant positive correlations were obtained separately and between variety and starch (0.565), respectively Kolbach index (0.641), Hartong index (0.616) and extract yield (0.891) between starch content and extract yield, with a coefficient of correlation r = 0.735.

Significant negative correlations were made separately between HM and protein content with a correlation coefficient r = - 0.771, between variety and protein content with a coefficient of correlation r = - 0.501, and significantly correlation between starch content and protein content r = - 0.395.

4. CONCLUSIONS

- The thousand grain weight (TGW) is a very important indicator for the quality of barley for brewing, positively correlated with all other quality indicators.
- Barley assortment influences positive all quality indicators except only protein content, which is negatively significantly distinctive correlated with this.
- Between Kolbach index and Hartong index there is a very significant positive correlation (0.999), while between protein content and starch content there is a significant negative correlation (- 0.395).
- Therefore should be created varieties of barley with a TGM higher and with higher assortment, in order to obtain quality malt.
- Tunika and Thuringia varieties have achieved the best results in our experience, cultivated on alluvial soil in Vădeni area.

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COMPARATIVE STUDY OF NEW INDETERMINATE TOMATO CULTIVARS IN VEGETABLE PRODUCTION AREA OF MATCA: QUALITY ASPECTS

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Abstract. *Two new tomato varieties (Lycopersicum esculentum Mill.), hybrids F1 (Amanda F1, Lady Rosa F1), with indeterminate growth, Israeli origin from Zeraim Gedera seed company have been tested for their quality characteristics compare with Menhir F1 (Netherlands, Nunhems seeds company) as control variety. The tests have been made in the experimental plots of SC MARCOSER SRL from vegetable production area of Matca, Galati County. For these tomato varieties was tested some agrochemicals parameters and biological factors in order to establish the quality of the tomato fresh fruit. Ascorbic acid levels were found between 12.7 and 15.1 mg per 100g of fresh weight in red ripe tomatoes and for lycopene the values vary from 19.1 to 23.5 mg/kg of fresh weight. The study shows that Lady Rosa F1 cultivar has the highest quality comparative with Amanda F1 and Menhir F1.*

Keywords: tomato hybrid, fruit quality, indeterminate, C vitamin, lycopene

1. INTRODUCTION

Fresh tomato and other tomato products make a significant contribution to human nutrition due to the concentration and availability of several nutrients in these products and to their wide spread consumption. Vitamin C, ascorbic acid, is a vitamin necessary for normal metabolism, wound healing and collagen synthesis. Lycopene is the most prominent carotenoid in ripe red tomatoes where it commonly constitutes around 90-99% of the total carotenoids (Dumas et al., 2003).

Influence of variety and differences in the amount of nutrients like potassium, vitamins, antioxidants contained in different varieties have been confirmed. In the research of Sahlin et al. (2004) it was shown that levels of C vitamin, Potassium and antioxidants vary between tomato varieties.

In last ten years was an increased interest from Romanian consumers for high quality tomatoes, quality expressed more by taste and nutritional factors then a perfect shape. These high exigent for quality demanded to test and introduce to the market new varieties to satisfy this shift of consumers expectation.

The yield capacity depends on the hereditary base of the cultivars and on the environmental conditions, including the conditions of culture (Gould, 1992). It also depends on the interaction between the genotype and the environmental conditions (Bletsos and Goulas, 2002; Susic et al., 2002)

Zeraim Gedera Seeds Company, which has ones of the most valuable genetic base and hybridation lines for tomatoes.

This work aimed to evaluate the quality of three tomato genotypes on titrable acidity (TA), pH, total soluble glucides, lycopene, ascorbic acid, and potassium, phosphates and nitrates contents in tomato.

2. MATERIALS AND METHODS

The experiments was made at the SC MARCOSER SRL experimental plots, in the biggest cold greenhouse vegetable production area in Romania - Matca village, located in Galati county in the East part of the country in the period January-June 2011.

For experiments were used two new indeterminate tomato varieties: Amanda F1 and Lady Rosa F1 form Zeraim Gedera, Israel compare with Menhir F1 from Nunhems Holland.

The technology of cultivation was standard with small particularities of Matca area technology: sowing in 10 January, planting in unheated greenhouse in 20 March, very early cycle of only 4 clusters.

The analysis was made during intense vegetative development of the fruits for nitrates, phosphates and potassium accumulation and after harvest for the rest of parameters.

3. RESULTS AND DISCUSSIONS

The results of analysis for nitrates, phosphates and potassium accumulation during vegetative development of tomatoes fruits are presented in Table 1:

Table1. Agrochemical composition of tomato fruits

No crt	**Variety**	$N\text{-}NO_3$, ppm	$P\text{-}PO_4$, ppm	K, ppm
05.06.2011				
1	Amanda	238,3	175,8	986
2	Lady Rosa	285,2	165,3	1102
3	Menhir	256,7	156,3	973
20.06.2011				
1	Amanda	204,0	198,7	1870
2	Lady Rosa	219,3	189,3	1890
3	Menhir	228,2	196,5	1950
30.06.2011				
1	Amanda	200,7	210,5	1503
2	Lady Rosa	198,3	215,3	1687
3	Menhir	204,3	205,4	1489

The content of nitrates calculated as average values per variety has oscillated from high values on the beginning of development (285,2 ppm at Lady Rosa F1) to lower values at harvest of the fruits (198,3 ppm at Lady Rosa F1). For all cultivars the nitrates quantities

at harvest period are lower than maximum quantities allowed by standards for fresh tomatoes fruits (300 ppm), the lower value being found for Lady Rosa F1.

Regarding phosphates and potassium it can be observed that the quantities are growing during development and have high values at harvest which means a very good quality of tomatoes especially in the case of Lady Rosa F1.

The results of biochemical parameters of tomatoes fruits are presented in Table 2.

Table 2. Biochemical results of tomatoes fruits

No crt.	**Variety**	TA, %	C Vitamin, mg/100g	Soluble glucides, %	Lycopene, mg/kg	pH
1	Amanda	0,510	12,625	4,798	19,118	4,650
3	Lady Rosa	0,578	15,100	4,550	23,350	4,375
2	Menhir	0,428	16,100	4,780	15,995	4,275

The tritable acidity varies from 0,428 % at Menhir F1 cultivar to 0,578% at Lady Rosa F1. The ascorbic acid levels in fresh fruits analyzed reveal that Menhir F1 has the highest value of 16,1 mg/100g fresh fruit and the low level it was found at Amanda F1 with 12,625 mg/100g fresh fruit.

Regarding the soluble glucides the most tasty cultivar is Amanda F1 with 4,798% concentration of soluble glucides and also this variety has the high pH of 4,65 compare with Lady Rosa F1 and Menhir F1 which have 4,375 respectively 4,275.

Regarding lycopene quantities the highest value was found at Lady Rosa F1 cultivar, this variety having the most intense red color compare with the other two cultivars.

4. CONCLUSIONS

In the case of agrochemical parameters analyzed it can be say that the nitrates quantities have decreased at all varieties tested and the lower value it was found at Lady Rosa F1 with 198,3 ppm.

Regarding phosphates and potassium accumulation, these two parameters being responsible for a good resistance at transport and shelf life of the fruits, all varieties accumulated, during vegetative development high quantities and the best results was found at Lady Rosa F1 cultivar.

From the point of view of biological factors analyzed all of varieties tested revealed good results. The high concentration in ascorbic acid was found at Menhir F1 and the highest concentration of glucides was found at Amanda F1 cultivar. Regarding total acidity and lycopene concentration the Lady Rosa F1 had the best results.

In conclusion all cultivars testes have the quality standards for human consumption and the best results were found at LADY ROSA F1 cultivar.

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STUDIES REGARDING THE EFFECT OF MULCHING WITH TRANSPARENT FILM IN GREENHOUSES OF MATCA VEGETABLE AREA ON QUALITY OF TOMATOES

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Abstract. *A study was carried out to determine the effect of mulching with transparent film compare to no mulching on quality of cv. AMANDA F1 tomato fruits (Lycopersicum esculentum Mill.) in cold greenhouses in vegetable area of Matca. Total nitrogen, total phosphate, total potassium, microelements, acidity, C vitamin, soluble glucide and lycopene were determined during the harvest period. The study shows that mulching had a positive effect on fruit quality.F1.*

Keywords: mulching, transparent film, cold greenhouse, tomato, quality

1. INTRODUCTION

The advantages of using plastic mulches for the production of high-value vegetable crops have been recognized since the late 1950s (Emmert 1957, Schales and Sheldrake 1965, Waggoner et al. 1960). Polyethylene (PE) mulches have induced large increases in growth and yields for a variety of crops, including tomato (TAKATORI et al., 1964; VANDENBERG & TIESSEN, 1972; MULLINS et al., 1992; LAMONT Jr., 1993). These growth and yield increases have been attributed to changes in soil and air temperature near the cover, soil water balance, and nutrient availability compared to unmulched soil (CLARKSON & FRAZIER, 1957; CLARKSON, 1960; BRUNINI et al., 1976; HAYNES, 1987).

In this study was analyzed the quality parameters of tomato fresh fruit in greenhouse with transparent film mulching compare with unmulched greenhouse.

2. MATERIALS AND METHODS

The experiments was made at the SC MARCOSER SRL experimental plots, in the biggest cold greenhouse vegetable production area in Romania - Matca village, located in Galati county in the East part of the country in the period January-June 2011.

There was used two greenhouses of 400 sqm (20mx20m), in one (V1) was installed transparent film on all soil surface and in second greenhouse wasn't installed the mulch film (M0).

In both greenhouses was planted a very early maturity, Amanda F1 tomato variety from Zeraim Gedera, Israel.

Distance between rows: 60 x 90 cm.

Distance between plants: 30 cm

Density: 45 000 plants/ha.

For experiment were monitored 10 plants in 3 random repetitions in both greenhouses.

The parameters analyzed were total nitrogen, total phosphate, total potassium, microelements, acidity, C vitamin, soluble glucide and lycopene for tomatoes fresh fruits after harvest.

3. RESULTS AND DISCUSSIONS

From Table 1 it can be seen that the fruits from V1 (the greenhouse with mulch film) accumulated more dry weight the M0 variant and also the conductivity is higher.

Table 1. Values for dry weight, pH and conductivity

Greenhouse	Dry weight (%)	pH	Conductivity (mS/cm)
M0 (without mulch)	6,8	4,25	5,1
V1 (with mulch	7	4,31	5,12

Regarding accumulation of total nitrogen, total phosphate and potassium there is difference of results as next: the total nitrogen is lower in V1 (0,2667%) than M0 (0,2386%) that suggesting a better final quality for the tomato fruits which are growing with mulch film. Regarding phosphate and potassium (Table 2) the results suggest a higher accumulation of these elements in the tomato fruit from the greenhouse with mulch film. A big concentration of potassium in tomato fruits will make a higher resistance of the fruit at transport and a longer shelf life.

Table 2. Total nitrogen, phosphate and potassium (% from dry weight)

Greenhouse	Nt (%)	P2O5 (%)	K2O (%)
M0 (without mulch)	0,2667	0,059	0,3
V1 (with mulch	0,2386	0,06	0,31

Regarding concentrations of microelements there are different results, if the concentration for Cu, Zn and Fe are lower in the greenhouse without mulching film for Mn, Mg and Ca the concentrations are lower in the greenhouse with plastic mulch (Table 3 and Table 4).

Table 3. Concentration of Cu, Zn, Mn and Fe ((% from dry weight)

Greenhouse	Cu (ppm)	Zn (ppm)	Mn (ppm)	Fe (ppm)
M0 (without mulch)	0,41	0,96	0,93	3,65
V1 (with mulch	0,65	1,19	0,91	5,36

Table 4. Concentration of Mg and Ca(% from dry weight)

Greenhouse	Mg (ppm)	Ca (ppm)
M0 (without mulch)	124,85	106,59
V1 (with mulch	118,4	79,4

Regarding biochemical parameters tested the results revealed that the levels of ascorbic acids and lycopene are higher in the mulched greenhouse compare with unmulched greenhouse (Table 5).

Table 5. Biochemical parameters

Greenhouse	Acidity, %	Ascorbic acid, mg/100gp.p.	Soluble glucides, %	Lycopene, mg/kg
M0 (without mulch)	0,56	12,4	4,81	13
V1 (with mulch	0,56	12,6	4,8	29,65

As it shows in Table 5 the soluble glucides concentration is higher in M0 and the acidity is the same in both experimental variants.

4. CONCLUSIONS

The results obtain in this study show that in both experimental variants can be produced tomato fruits which has the quality for human consumption.

With small exceptions regarding some microelements and soluble glucides the rest of agrochemical and biochemical analyses made in this study reveal that growing tomatoes in greenhouses with transparent mulch film has a positive influence on the quality of fresh fruit tomatoes.

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THE INFLUENCE OF RIPENING TEMPERATURE ON DIVERSITY OF NON-STARTER LACTIC BACTERIA IN SEMI-HARD CHEESES

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Key words: NSLAB, cheese, ripening, *Lactobacillus spp.*

The role of non-starter lactic bacteria (NSLAB) in determination of cheese quality is still unclear. The only way how to ensure quality of cheese is corrections in technological process. The aim of the study was to evaluate impact of the selected ripening temperature on the growth rate of NSLAB in Latvian semi-hard Holandes and Krievijas cheeses.

The samples of unripened semi-hard cheeses have been analysed. Cheeses were produced at two cheese factories and ripened at laboratories of LUA for 60 days at 6 and 12 °C. Both ripening regimes help understand better the influence of microflora on the quality of cheese, including the formation of sensory properties.

The following analyses are performed in the study: pH, water activity (a_w), CFU of Lactobacillus spp., identification of Lactobacillus species and isolation of DNA for confirmation of isolated Lactobacillus species. The obtained sequences are analysed at Staden Package 1.6.0 release and compared to sequences available in the BLAST (www.ncbi.nlm.nih.gov.)

In order to control the rate of cheese ripening and the growth dynamics of mesophilic NSLAB, some researchers have suggested decreasing of ripening temperature. Decreasing of ripening temperature slows down the growth rate of mesophilic NSLAB, but it is impossible to liberate cheese from their presence. The differences are observed in qualitative composition of cheese microflora. L.curvatus dominated during the rest of the ripening time at 6 °C and L.plantarum 1 was detected at 12°C in cheese samples. DNA fragment sequencing of most frequently identified L.plantarum 1 and L.curvatus revealed that nucleotide sequence of L.curvatus of ripened cheese at 6°C for 60 days conforms with the strain L.paracesei subsp. paracasei JCM8133; in cheese ripened at 12 °C for 60 days - L.paracasei MH55. In their turn, the isolated L.plantarum 1 from cheese samples ripened at 12 °C for 60 days conform to L.plantarum DSPV 354T.

This study reveals that the concentration of NSLAB differs between cheeses ripened at 6 °C (5.26-6.90 log CFU g^{-1}) and 12 °C (5.26-7.62 log CFU g^{-1}) at least by 1 log. Higher concentrations were found in cheeses ripened at 12 °C. These findings should be taking account, because the temperature at ripening is subordinated to the rate of biochemical processes in manufacture of a particular cheese variety. Any change has a significant impact on the whole complex of sensory properties of cheese.

1. INTRODUCTION

Raw milk is a natural growth medium for microorganisms. Composition and quality of milk microflora are determined not only by hygiene observation in the places of milk production, rapidity of milk cooling and temperature, but also by microflora in the air of dairy environment, on the surface of equipments and

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premises. An integral part of milk microflora is non-starter lactic acid bacteria. Pasteurization regime selected in cheese manufacturing is able to destroy essential microflora, enzymes and pathogens in milk. It should be noted that inactivation level of microorganisms depends on the count of microorganisms, growth phase and other factors. Bactofugation, microfiltration, and application of food additives, cannot significantly decrease the proportion of *Lactobacillus spp*. and *Leuconostoc spp.* in milk. Defects caused by non-starter lactic acid bacteria are found in all dairy products, but the most problematic they are in cheeses.

Early blowing caused by non-starter lactic acid bacteria is often mixed up with coliform bacteria. Non-starter lactic acid bacteria, mostly heterofermentative, produce diacetyl and acetoin, and high amounts of carbon dioxide. Carbon dioxide forms many small holes in cheese, and sometimes a significant gas pressure results in a sponge-like cheese texture. Identical defect is caused by coliform bacteria. This defect occurs during the beginning of cheese ripening while there is lactose in it. Technological methods to fight coliform bacteria differ as well. If milk is produced according to hygienic requirements, food additives are not necessary for decreasing of activity of coliform bacteria. Over consumption of sodium nitrate/sodium nitrite is not beneficial for consumers. Cheesemakers are confused about the defect, its origin and possible solution. Enzymes produced by *Lactobacillus* spp. are able to hydrolyze casein forming bitter peptides, which influence flavour characteristics of cheese.

The non-starter lactic acid bacteria could not be evaluated unambiguously. Individual strains of species are used for acceleration of cheese ripening, stimulation of hydrolysis of proteins and enhancement of concentration of free amino acids contributing to flavour and aroma of a well ripened cheese. It should be noted that the role of non-starter lactic acid bacteria in determination of the cheese quality is still unclear. The aim of the study was to evaluate impact of the selected ripening temperature on the growth rate of NSLAB in Latvian semi-hard Holandes and Krievijas cheeses.

2.MATERIALS AND METHODS

The samples (n=18) of unripened semi-hard Krievijas and Holandes cheeses have been chosen for analysis. Cheeses were produced at two cheese factories in Latvia and ripened at laboratories of Latvia University of Agriculture. Cheeses are manufactured according to the technical requirements of the enterprise or the Branch Standards: LPCS 10: 2001 and LPCS 11: 2001. The technology of

cheeses is related to Gouda type cheeses, there are some differences regarding to added amount of starter, pH of grains and whey mixture before heating for Holandes cheese and prolonged pressing before brining of Krievijas cheese for intensification of lactic acid fermentation process.

The samples of unripened cheese were packed in a polymer material film, and ripened for 60 days at 6 °C and 12 °C. Ripening temperature was chosen taking as a basis conclusions in research articles relating variability of microflora (6 °C), and the recommended ripening parameters (12 °C) for Krievijas and Holandes cheeses. The both ripening regimes help understand better the influence of microflora on the quality of cheese, including the formation of sensory properties.

Determination of the pH was performed to cheese samples, according to LVS ISO 5546:2010 'Caseins and caseinates – determination of the pH', by using pH-meter, 3520 pH Meter'-JENWAY (Barloworld Scientific Ltd., Essex, UK).

Determination of water activity was performed for cheese samples, by using Meter AquaLab LITE (Decagon Inc, USA). Samples for determination of water activity were measured with accuracy ± 0,015. Prior to sample analyzing calibration of the equipment was performed by means of 0.5M KCl (Lot 932375, Decagon).

Determination of Lactobacillus spp. was performed in analysed samples, according to LVS ISO 15214:1998 by using MRS agar media (Scharlau, Spain). Media was prepared according to LVS CEN ISO/TS 11133-1:2009. Sample dilutions were performed according to LVS EN ISO 8261:2002 and ISO 6887-5:2010.

Colony forming units of the lactic acid bacteria were determined by means of ISO 15214:1998. The chosen parameters for cultivation of lactic acid bacteria in MRS agar were 72 hours at 37 °C, taking as a basis regimes recommended in the scientific literature (Coeuret et al., 2003).

Identification of Lactobacillus spp. colonies was performed taking as a basis fermentation of carbohydrates by using API 50 CHL (BioMerieux, France). The program APILAB Plus version 4.0 (BioMerieux) was used for identification of the isolated colonies up to species.

Isolation of DNA was performed for most frequently identified representatives of *Lactobacillus* spp. species - *L.plantarum 1, L.curvatus*, by using PowerSoil DNA Isolation Kit (MO BIO Laboratories Inc.).

Polymerase chain reaction analysis was performed for confirmation of the isolated *Lactobacillus* species. The obtained sequences were analyzed at *Staden Package 1.6.0. release* (http://staden.sourceforge.net/) and compared to sequences available in the data base BLAST (www.ncbi.nlm.nih.gov *).*

Data analyse was performed by using StatistiXL and Microsoft Excel programs.

3. RESULTS

In the beginning of cheese ripening the concentration of starter lactic acid bacteria decreases rapidly, thus liberating space for multiplication of non-starter lactic acid bacteria.

In the Tables 1, summarization about the lactic acid bacteria species isolated from Krievijas cheese is provided, as well as variability of species under the influence of temperature and duration of ripening is given.

In the beginning of ripening Krievijas cheese II presence of starter microflora was detected and prevalence of ***L.curvatus*** until the 30th ripening day at temperature 6 °C was observed. From a sample ripened for 45 days at temperature 6 °C *L.helveticus* was isolated. Williams and Banks (1997) report on isolation of *L.helveticus* from Cheddar cheese ripened for 6 – 9 months, considering this species as non-starter lactic acid bacteria. During ripening of Krievijas cheese I and II at temperature 12 °C, a presence of ***L.plantarum*** 1 was detected.

Table 2

The changes of *Lactobacillus spp.* during Krievijas cheese ripening

Ripening time, days	at 6°C		at 12°C	
	Krievijas cheese I	Krievijas cheese II	Krievijas cheese I	Krievijas cheese II
Unripened	*Lc.lactis* subsp.*lactis 1*, *L.curvatus*	*Lc.lactis* subsp.*lactis 1*, *Lc.lactis* subsp.*lactis 2*, *L.curvatus*	*Lc.lactis* subsp.*lactis 1*, *L.curvatus*	*Lc.lactis* subsp.*lactis 1*, *Lc.lactis* subsp.*lactis 2*, *L.curvatus*
15 days	*Lc.lactis* subsp.*lactis 2*, *L.curvatus*		*L.curvatus*	*L.plantarum 1*
30 days	*L.curvatus*	*L.curvatus*		
45 days		*L.helveticus*		*L.plantarum* S4
60 days		*L.paracasei* subsp. *paracasei* JCM 8133		*L.paracasei* MH55, *L.plantarum* DSPV 354T

DNA fragment sequencing of most frequently identified ***L.plantarum 1*** and ***L.curvatus*** revealed that nucleotide sequence of ***L.curvatus*** of ripened cheese for 60 days at 6 °C conforms to the strain ***L.paracasei*** subsp.***paracasei*** JCM 8133, but in cheese ripened for 60 days at 12 °C – to the strain ***L.paracasei*** MH55. In their turn, the isolated ***L.plantarum 1*** from cheese samples ripened for 45 and 60 days at 12 °C conform to the strains ***L.plantarum*** S4 and ***L.plantarum*** DSPV 354T respectively.

API 50 CHL system applied for identification of *Lactobacillus* phenotypically showed satisfactory results when determining genus of microorganisms. According to Tynkkynen (1999) and co-authors, its precision is considerably lower when identifying microorganisms up to species. This could be explained by the fact that the system initially was intended for identification of

Lactobacillus genus for medical needs (Coeuret et al., 2003) as a supplementary system for atypical fermentation models (Arhné et al., 1989; Chamba, 2000; Coeuret et al., 2003). Muyanja (2003), Temmerman (2004) and co-authors concluded that the phenotypical methods are limited in respect of reproductivity, with low taxonomic resolution, and they often allow identification only at the level of genus.

Table 3

The changes of *Lactobacillus spp.* during Holandes cheese ripening

Ripening time, days	at 6°C		at 12°C	
	Holandes cheese I	Holandes cheese II	Holandes cheese I	Holandes cheese II
Unripened	*Lc.lactis* subsp.*lactis 2*, *Lc.lactis* subsp.*lactis 1*	*Lc.lactis* subsp.*lactis 1*, *Lc.lactis* subsp.*lactis 2*	*Lc.lactis* subsp.*lactis 2*, *Lc.lactis* subsp.*lactis 1*	*Lc.lactis* subsp.*lactis 1*, *Lc.lactis* subsp.*lactis 2*
15 days	*Lc.lactis* subsp.*lactis 1*, *L. paracasei* subsp.*paracasei 1*		*L. paracasei* subsp.*paracasei 2*, *L.rhamnosus*	*Lc.lactis* subsp.*lactis 2*, *L. paracasei* subsp.*paracasei 1*
30 days	*L. paracasei* subsp.*paracasei 2*, *L.curvatus*	*L. paracasei* subsp.*paracasei 1*, *L. paracasei* subsp.*paracasei 3*	*L. paracasei* subsp.*paracasei 1*, *L. paracasei* subsp.*paracasei 2*	*L.plantarum 1*
45 days	*L. paracasei* subsp.*paracasei 1*, *L. curvatus*	*L. paracasei* subsp.*paracasei 3*, *L.curvatus*	*L. paracasei* subsp.*paracasei 1*	
60 days	*L. paracasei* subsp.*paracasei 2*	*L.curvatus*	*L. paracasei* subsp.*paracasei 1*, *L.rhamnosus*	

In the unripened Holandes cheese I and II presence of starter microflora was detected (Tab. 2). In Holandes cheese I, starting from the 15th day of ripening until the end of ripening, *L. paracasei* subsp.*paracasei* dominated. From the sample ripened at temperature 6 °C, *L.curvatus* was isolated as well, but from the sample ripened at 12 °C - *L.rhamno* Holandes cheese II domination of *L. paracasei* subsp.*paracasei* was observed until the 45th day, ripening at temperature 6 °C, and prevalence of *L.plantarum 1* from the 30th until the 60th day of ripening, at temperature 12 °C. According to the data by Copolla et al. (1997) and Fitzsimons (1999), most frequently *L.casei*, *L.paracasei*, *L.plantarum*, *L.rhamnosus* and *L.curvatus* are isolated from cheeses.

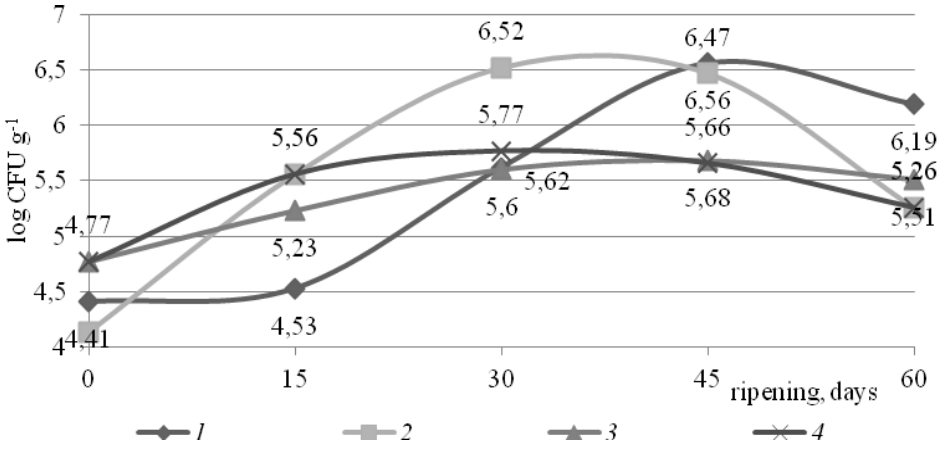
According to the findings by Fitzsimons et al. (2001) and Williams et al. (2002), the dominating non-starter lactic acid bacteria species usually change during the cheese ripening, and are represented by one *Lactobacillus* species by the end of ripening. In the opinion of these authors, *L.paracasei*, *L.plantarum* and *L.brevis* dominate in unripened cheeses, but *L.paracasei* – in ripened cheeses.

Diversity of microflora and its growth intensity in analyzed cheeses depend on water activity, salt content, pH, ripening temperature and oxidation-reduction potential. Therefore the dynamics of the colony forming units of non-starter lactic acid bacteria under the influence of some factors is evaluated.

Quantity of microflora is characterized by the count of colony forming units of lactic acid bacteria in experimental Krievijas (Fig. 1) and Holandes (Fig. 2) cheeses.

Non-starter lactic acid bacteria growth rate in the beginning of ripening was observed in Krievijas cheeses I and II, moreover, the growth rate was higher at 12 °C than at temperature 6 °C. More rapid growth rates are explained by temperature closer to optimum of the mesophilic bacteria activity and availability of nutrients.

Shakel-Ur-Rehman (2000) and co-authors report that in cheese ripened at temperature 1 °C, population of the non-starter lactic acid bacteria is for 3 log less than in cheese ripened at 8 °C. Other authors have similar opinions that ripening temperature influences considerably the growth of non-starter lactic acid bacteria in cheese (Folkertsma et al., 1996; Fenelon et al., 1999).

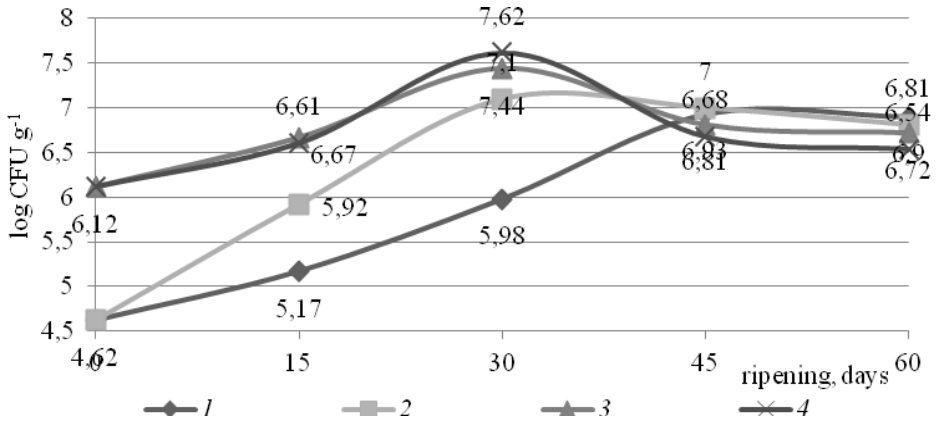


1-Krievijas cheese I, 6°C; 2-Krievijas cheese I, 12°C; 3-Krievijas cheese II, 6°C; 4-Krievijas cheese II, 12°C

Figure 1. **The dynamics of colony forming units of non-starter lactic acid bacteria in Krievijas cheeses during ripening**

When analyzing Holandes cheeses, similar situation was observed. *Lactobacillus* spp. its maximum concentration in Holandes cheese I and II reached around the 30th day when ripened at 12 °C, and around the 45th day, when ripened at 6 °C. Changes in the count of colony forming units of non-starter lactic acid

bacteria show that their increase depends on temperature at ripening, composition and characteristics of the starter, availability of nutrients and other factors.

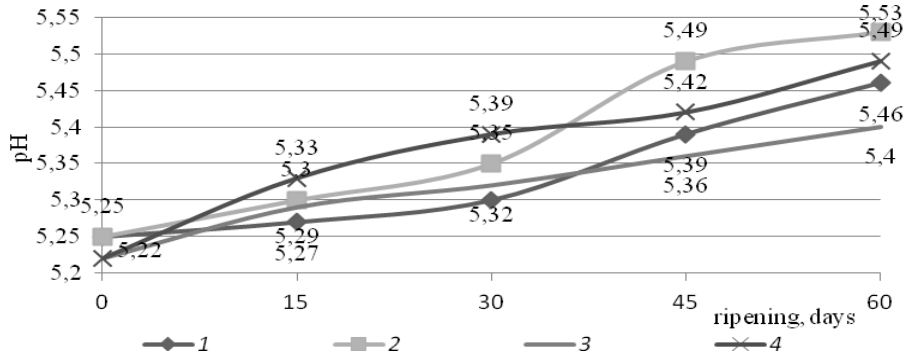


1-Holandes cheese I, 6°C; 2-Holandes cheese I, 12°C; 3-Holandes cheese II, 6°C; 4-Holandes cheese II, 12°C

Figure 2. **The dynamics of colony forming units of non-starter lactic acid bacteria in Holandes cheeses during ripening**

pH is one of the most important factors during cheese ripening, enhancing activity of enzymes and regulating the growth of microorganisms. Due to production technology peculiarities of Krievijas cheese, the pH in cheese after pressing rises up to 5.2 – 5.3. The most part of lactose is fermented till brining, creating characteristic acidity of Krievijas cheese (pH 5.22 – 5.25), which corresponds to the results of Krievijas cheeses I and II (Fig. 3), respectively, pH 5.25 and 5.22.

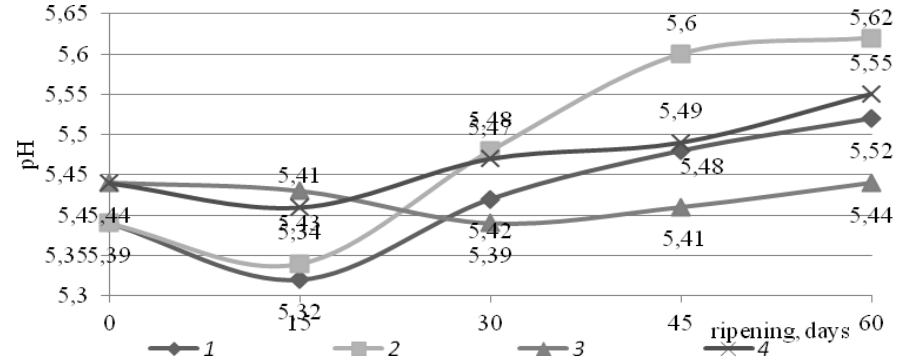
Increase of pH in Krievijas cheeses during the beginning of ripening up to the 15th day must be explained by continuation of lactic acid fermentation and use of lactic acid in further chemical processes. Dispersion analysis revealed significant differences ($p<0.05$) between Krievijas cheeses ripened at 6 and 12 °C.
When observing changes of the pH in Holandes cheeses I and II (Fig.4), the conclusion follows that during the first 15 days of ripening the pH decreases irrespective of temperature chosen at ripening. As indicated in different sources, the pH of Holandes cheeses rises up to 5.2 – 5.25 after 3 days of ripening. Differences could be explained by the peculiarities of technological process, the type of used starters and the quality of milk.



1-Krievijas cheese I, 6°C; 2-Krievijas cheese I, 12°C; 3-Krievijas cheese II, 6°C; 4-Krievijas cheese II, 12°C

Figure 3. **The dynamics of pH in Krievijas cheeses during ripening**

In order to make sure of influence of the pH on the water activity, correlation analysis of the mentioned parameters was performed. The obtained results reveal a strong negative linear correlation (Krievijas cheeses r=0.87; Holandes cheeses r=86) between the parameters under study. This supports opinion of several authors (Fox et al., 2000; Folkertsma et al., 1996; Aston et al., 1985) that during cheese maturation while the amount of water-soluble nitrogen compounds and the use of lactic acid increase, the rise of the pH value and reduction of water activity takes place. Water activity in experimental Krievijas cheeses during ripening varied from 0.994 up to 0.960, but in Holandes cheeses from 0.995 up to 0.971.



1-Holandes cheese I, 6°C; 2-Holandes cheese I, 12°C; 3-Holandes cheese II, 6°C; 4-Holandes cheese II, 12°C

Figure 4. **The dynamics of pH of Holandes cheeses during ripening**

In order to control the rate of cheese ripening and the growth dynamics of mesophilic non-starter lactic acid bacteria, some researchers have suggested decreasing of ripening temperature. Decreased ripening temperature slows down the growth rate of mesophilic non-starter lactic acid bacteria, but it is impossible to liberate cheese from their presence. Also this study reveals that the concentration of non–starter lactic acid bacteria differs between cheeses ripened at 6 and 12 ° C at least by 1 log. Higher concentrations were found in cheeses ripened at 12 ° C. These findings should be taking into account, because the temperature at ripening is subordinated to the rate of biochemical processes in manufacture of a particular cheese variety. Any change has a significant impact on the whole complex of the sensory properties of cheese.

4. CONCLUSIONS

1. Identified *Lactobacillus* spp. well-adapted to variable parameters of cheese ripening, and their population and growth rate are dependant on diversity of substrate in cheeses.
2. The prevalence species of non-starter lactic acid bacteria in cheeses varies during ripening and at the end of ripening were represented by one species of *Lactobacillus* genus, more often *L.curvatus, L.paracasei* subsp.*paracasei* or *L. plantarum.*
3. Diversity of lactic acid bacteria species in Krievijas and Holandes cheeses depends on selected ripening temperature and time. Representatives of *Lactobacillus* genus and its colony forming units differs between same variety cheeses manufactured at different plants.
4. The close correlation was determined between changes of pH and a_w in analyzed Krievijas and Holandes cheeses. This indicates intensity of biochemical and microbiological processes during ripening.

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