ISSN: 2490-3442

UNIVERSITY OF EAST SARAJEVO
FACULTY OF AGRICULTURE

AGROFOR International Journal

AGROFOR International Journal, Vol. 4, Issue No. 3, 2019

agrofor.ues.rs.ba

AGROFOR International Journal

PUBLISHER
University of East Sarajevo, Faculty of Agriculture
Vuka Karadzica 30, 71123 East Sarajevo, Bosnia and Herzegovina
Telephone/fax: +387 57 340 401; +387 57 342 701
Web: agrofor.ues.rs.ba; Email: agroforjournal@gmail.com

Frequency: 3 times per year
Number of copies: 300
ISSN 2490-3434 (Printed)
ISSN 2490-3442 (Online)

CONTENT

Original Scientific paper
10.7251/AGRENG1903005P
UDC 633.15:557.2

DIFFERENTIATION OF MAIZE LINES WITH HIGH CONTENT OF CAROTENOIDS USING PROTEIN AND DNA MARKERS

Larysa PRYSIAZHNIUK[1*], Yurii HONCHAROV[2], Yuliia SHYTIKOVA[1], Oksana TOPCHII[1], Snizhana OTROSHKO[1]

[1]Laboratory of Molecular Genetic Analysis, Ukrainian Institute for Plant Variety Examination, Ukraine
[2]Research Institute of Agrarian Business, Ukraine
*Corresponding author: prysiazhniuk_l@ukr.net

ABSTRACT

Wide natural variety of carotenoids, including vitamin A precursors, is characteristic of maize (Zea mays L.), which allows using it to combat vitamin A deficiency in the world. Previous studies have established the effectiveness of the use of functional DNA markers in the selection of maize lines with a high content of carotenoids in grain. However, not only improving grain quality but also creating highly productive hybrids competitive on the grain market is currently important. The purpose of our study was to determine the genetic diversity of maize lines using storage protein and DNA markers, as well as to find correlations of two marker systems with FAO characteristics. On the basis of maize lines selected for high content of carotenoids, the allelic state of six SSR markers (phi022, phi034, phi062, phi073, phi079, phi085), electrophoretic spectra of zein and their electrophoretic mobility have been determined. Cluster analysis of maize lines using electrophoretic spectra of zein yielded eight clusters. It was found that the minimum genetic distance was 4.24 and the maximum 7.48 Cluster analysis by the identified alleles for SSR markers allowed to form seven clusters according to the affinity of the lines. Range of changes in genetic distances was from 1.00. to 3.46 The analysis of genetic distance matrices, using the Mantel test, found a correlation between the marker systems under study (r = 0.184). A correlation between the studied marker systems and their relation to FAO characteristics was established. Therefore, in order to increase selection efficiency of maize, it is advisable to use an integrated approach to the evaluation of breeding genotypes involving protein and DNA markers.

Keywords: *storage proteins; SSR markers; cluster analysis; correlation.*

INTRODUCTION

Maize (*Zea mays* L.) is represented by a sufficiently variable gene pool that allows using of different genotypes in breeding, which contributes to improvement of the hybrids' productivity in terms of crop productivity, disease resistance, duration of

the vegetation season and other agronomic characteristics. Various approaches are used for the corn genetic diversity assessment, which include the assessment of both morphological characteristics and the molecular genetic analysis of genotypes. Previous studies have shown the usage of SSR markers and electrophoretic spectra of maize seeds' reserve proteins for evaluation of maize lines' genetic diversity of domestic breeding and well-known lines (Goncharov *et al*., 2016; Prysiazhniuk *et al*., 2018), as well as usage of DNA markers for the selection of lines with high carotenoids content (Prysiazhniuk *et al*., 2019). Polymorphism of seeds' reserve proteins allows estimation of inbred lines for genetic homogeneity through the component composition of zein spectra, as well as with a high probability indicates the degree of genetic proximity between lines (Pedersen *et al.*, 1982; Feix, and Quayle, 1993). Studies by Sidorova *et al.* (2012, 2015) identified the presence of certain components of zein in maize lines from different maturity groups and identified components, which are distinctive for early-maturing maize with FAO till 299. Such studies are essential in context of searching of optimal parental components for creation of the highly productive hybrids that can be domesticated in different soil-climatic zones.Papers devoted to searching for microsatellite loci related to the agronomic characteristics were carried out by many authors (Lu and Bernardo, 2001; Legesse *et al.*, 2007). Magulama and Sales (2009) and Yang *et al.* (2008) have described usage of phi 057 and phi 112 markers for assessing of maize genotypes with high levels of lysine and tryptophan. Thus, the interest appears for the application efficiency of zein and SSR markers for evaluation of a small sample of lines with specific characteristic such as high carotenoid content, as well as comparing the efficiency of marker systems for lines differentiation. Furthermore, the issue of assessing the correlation connections between SSR markers and maize maturity groups remains to be underinvestigated.

Consequently, the purpose of our study is to determine the genetic diversity of maize lines with high content of carotenoids in grain with usage of storage proteins and DNA markers, as well as to find correlations between two marker systems with FAO indexes.

MATERIAL AND METHODS

Materials for study were 21 lines of maize with high content of carotenoids. Studied maize lines had the following FAO indexes: DK129-4 – 170, DK366 –190, DK2323 – 190 (early-season group); DK959 – 200, DK247 – 210, DK212 – 220, DK267 – 220, DK273 – 220, DK744 – 220, DK276 – 230, DK272 – 240, DK239 – 250, DK742 – 280, DK680 – 280, DK296 – 280 (middle-early group); DK633/266 – 300, DK257 – 3203 (mid-season group); DK411 – 400, DK325 – 400, DK377 – 430, DK633 – 450 (middle-late group). Maize lines were selected based on DNA markers and on total carotenoid content in grain (Prysiazhniuk *et al.*, 2019). The research was performed during 2016-2018 on the basis of the laboratory of biotechnology in the state-owned institution Institute of grain crops of NAAS within the framework of the State Program of Scientific Research 23 "Biotechnology and Genetics in Crop Farming" task 23.00.01.06F "Development

of the Fundamental Basics of Molecular, Genetic and Cell Biotechnologies for the Improvement of Maize Selection" and the department of laboratory tests for the qualifying expertise of plant varieties (Center for Certification Tests) in the Ukrainian Institute of Plant Varieties Expertise. Maize lines polymorphism was investigated based on protein and DNA markers. Electrophoretic spectra of storage proteins were evaluated based on the electrophoretic mobility of zein components (rf) (Prysiazhniuk *et al.*, 2018). For the determination of polymorphism of the six maize lines according to DNA markers six SSR markers (phi022, phi034, phi062, phi073, phi079, phi085) were used (Goncharov *et al.*, 2016). The magnitude of electrophoretic mobility and the size of alleles were determined using TotalLab TL 120 software (trial version). In accordance with the obtained data values of electrophoretic mobility, the frequencies of the identified zein components were calculated according to the formula:

$$F_i = \frac{n_i}{N},$$

where *Fi* – frequency of the *i* component of zein, n_i – number of the *i* component in the sample, *N* – total number of maize lines.

According to SSR markers, allele frequencies and the polymorphic index of the locus (RIS) were determined (Sivolap *et al.*, 1998). Determination of genetic distances between maize lines were estimated with the help of cluster analysis. The unweighted pair-group average method was used as an amalgamation (linkage) rule (Fortin *et al.*, 2002; Everitt *et al.*, 2011). Estimation of the connections of FAO indexes of the studied maize lines with DNA markers was performed using the Pearson linear correlation method, with protein markers, Spearman nonparametric statistical methods. Statistical data was calculated using STATISCA 12 computer program (Trial version) (Johnson and Wichern, 2002; Elliott and Woodward, 2007). The evaluation of the correlation between two marker systems by genetic distances was performed using the Mantel test using the XLSTAT 2018 (Trial version) computer program (Legendre *et al.*, 2010; Diniz-Filho *et al.*, 2013).

RESULTS AND DISCUSSION

As the result of polymorphism study of 21 maize lines according to zein spectra were identified from 12 to 18 components for each line. It was determined that 9 components were unique and identified in one-off event in the studied lines. The electrophoretic mobility (Rf) of these components was between 32 and 102.

According to the obtained distribution, components with Rf 32 and 77 identified in DK129-4 line. It was specified that component with Rf 35 is distinctive for DK2323 line, and component with Rf 102 - for the DK325 line. Also, the unique components were identified in lines DK247 and DK267, Rf 62 and Rf 96, respectively. Components with Rf 70, Rf 78 and Rf 95, which were also found only once, were identified in the DK276 line. In order to assess the similarity of the studied maize lines a cluster analysis carried out according to the spectra of storage proteins in order to determine the genetic distances between the objects of analysis. The results of the hierarchical classification as a phylogenetic tree is represented in Figure 1.

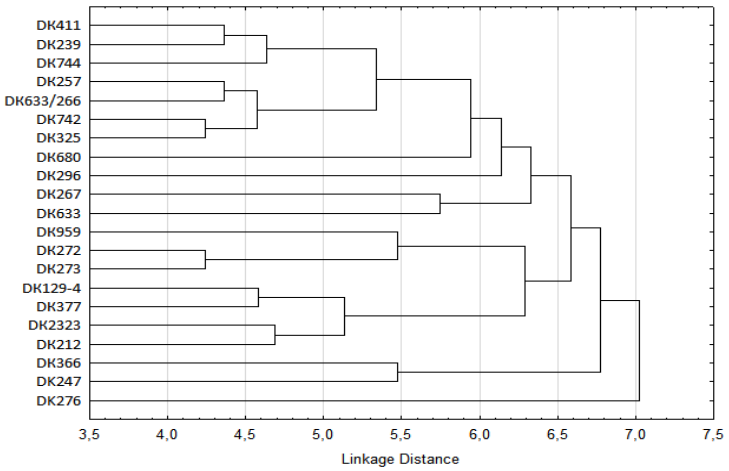

Fig. 1. Distribution of maize lines according to the degree of affinity on the basis of electrophoretic separation of zeines

In the result of cluster analysis, 8 clusters were obtained, which were formed by the maize lines according to the spectra of storage proteins. According to the received distribution, the most similar were lines included in the same cluster: DK742 and DK325, DK272 and DK273 with a genetic distance between them 4.24. It was determined that lines with the smallest distance between them was different according to 5-6 components. Thus, in DK742 line, components with Rf 55, 68, 83 and 101 were discovered, and in DK325 line components with Rf 66, 82, 88, and 102 were identified. It was also found that in the other pair of the most similar lines DK272 and DK273 determined components with Rf 46, 66, 72, 76, 80, 89 and Rf 47, 73, 81 respectively, which differed them from each other.

The most distant in relation to other lines were DK296 and DK276. It was determined that the values of genetic distances between DK276 and DK633/266 lines and DK272 varied from 6.0 to 7.48, respectively.

The research established that zeines are inherited by electrophoretic units: components with average electrophoretic mobility, components that are grouped into a block of polypeptides with the largest and smallest electrophoretic mobility, and components that are independently inherited (Zayakina and Sozinov, 1993; Zayakina and Sozinov, 1997; Zayakina *et al*., 1998). According to the Spearman statistical analysis, a correlation between components with average electrophoretic mobility and with its maximum and minimum values was determined. Thus, the correlation coefficient between components with Rf 56 and Rf 86 was 0.68. The statistically significant correlation coefficients between Rf 62 and Rf 84, Rf 48 and Rf 74, Rf 72 and Rf 80 were also determined and were 0.69, 0.70 and 0.71, respectively. It was determined that the correlation coefficient between components with Rf 70 and Rf 78 was 1.00, which indicates a close correlation.

Our research also confirmed the existence of correlation between components with maximum and minimum electrophoretic mobility. Thus, the existence of a close

connection (1.00) between components with Rf 32 and Rf 77 was determined. It was found that the correlation coefficients between Rf 42 and Rf 99 were 0.56, between Rf 40 and Rf 100 – 0.61, and correlation between components with Rf 39 and Rf 92 – 0.52 and between Rf 49 and Rf 91 the correlation coefficient was 0.74. Studies that identified the complex organization of zein encoding genes were performed by Hagen and Rubenstein (1981) by analysis of restriction fragments and southern blot hybridization, and confirmed by Zayakina and Sozinov (1993) by means of electrophoregram analysis of prolamins. Therefore, according to our research, a very strong, strong and significant correlation between zein components with different electrophoretic mobility was determined.

According to the results of our work, polymorphism of maize lines according to SSR markers was also determined. The electrophoregrams of the studied maize lines according to the markers phi034 and phi085 are shown in Figures 2 and 3.

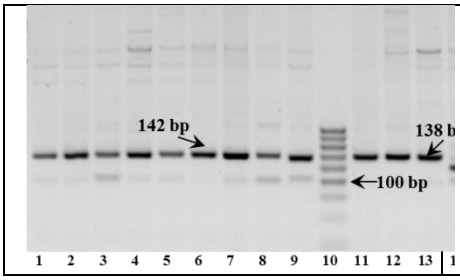

Fig. 2. Electrophoresis of amplification products of maize DNA with a marker phi034: 1 – DK411; 2 – DK257; 3 – DK742; 4 – DK744; 5 – DK325; 6 DK633/266; 7 – DK680; 8 – DK296; 9 – DK267; 10 – molecular weight marker 20 bp DNA Ladder O'GeneRuler (Thermo Scientific, USA); 11 – DK633; 12 – DK366; 13 – DK247; 14 – DK276; 15 – DK959

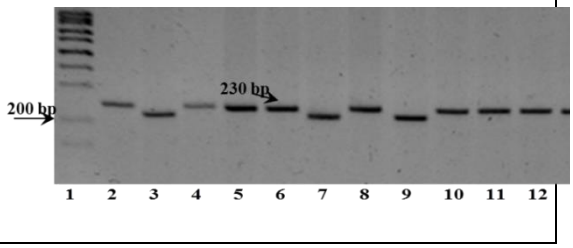

Fig. 3. Electrophoresis of maize DNA amplification products with marker phi085: 1 – molecular weight marker 100 bp DNA Ladder O'GeneRuler (Thermo Scientific, USA); 2 – DK296; 3 – DK267; 4 – DK633; 5 – DK366; 6 – DK247; 7 – DK276; 8 – DK959; 9 – DK272; 10 – DK273; 11 – DK129-4; 12 – DK377; 13 – DK2323; 14 – DK239

As can be seen from Figure 2, alleles with a size 118, 138, 142 and 147 bp was identified according to the phi034 marker. The frequencies of investigated alleles were 0.10-0.48, PIC – 0.66. The phi085 marker identified six alleles, the size of which ranged from 213 to 252 bp (Fig. 3). It was determined that allele with the size 252 bp identified in the DK212 line was specified as unique for studied lines according to phi085 marker.

The most common allele was 230 bp, which identified in 8 out of 21 studied lines. The frequency of the identified alleles according to the phi085 marker is 0.05-0.38, the PIC is 0.83. All alleles, which were determined in the studied lines and the PIC values, represented in Table 1.

Table 1. Alleles identified by SSR markers and their PIC

Name of markers	Alleles size, bp	Alleles frequency	PIC
phi034	118; 138; 142; 147	0.10-0.48	0.66
phi062	143; 148; 155	0.05-0.90	0.18
phi073	174; 178; 182; 187	0.14-0.48	0.70
phi079	171; 176; 183; 189	0.10-0.62	0.59
phi022	125; 130; 155; 160	0.10-0.62	0.60
phi085	213; 220; 230; 236; 241; 252	0.05-0.38	0.83

The size of alleles according to the phi085 marker in our studies coincided with the study results of North American maize germplasm investigation published in Maize Genetics and Genomics DataBase (MaizeGDB). Smith *et al.* (1997) conducted research with 58 inbred lines and 4 maize hybrids according to 131 SSR markers with the purpose to identify lines and evaluate genetic distances between them. With markers phi022 and phi062, authors obtained two alleles for each marker; the PIC was 0.46 and 0.48, respectively. In our studies, these markers identified from 3 to 4 alleles, with a PIC value 0.60 and 0.18 for markers phi022 and phi062, respectively. In the result of Sharma *et al.* (2010) studies, by means of phi062 marker four alleles were received, and the PIC was 0.18, which also coincides with our results. In order to determine the ability of investigated SSR markers to differentiate the maize lines, they performed a cluster analysis according to the presence/absence of particular size of alleles (Fig. 4).

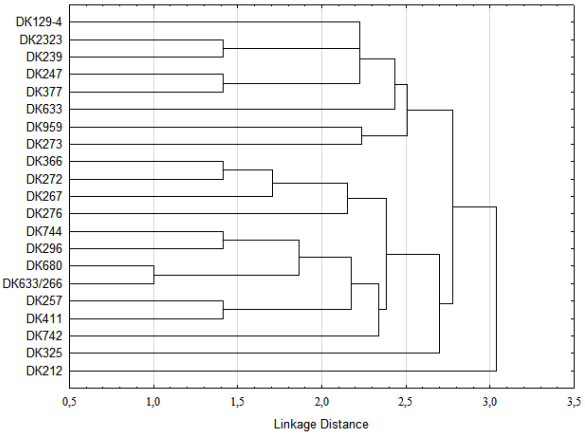

Fig. 4. Distribution of maize lines according to the degree of affinity based on SSR markers

In consequence of the maize lines distribution according to SSR markers, seven clusters were obtained according to the genetic distances between the lines. It was found that the most similar were the lines included in one cluster DK680 and DK633/266, the value of genetic distances between them was 1.0. The most distant lines that formed the same cluster were lines DK959 and DK273 (genetic distances 2.24). According to the obtained data, the most distant line was DK212. The value of genetic distance towards to other studied lines was from 2.83 to 3.46. Accordingly, the most distinguished lines were DK212 and DK2323, genetic distances between which were the strongest. The DK325 line was also not included in any cluster and was at a distance of 2.45-3.16. Consequently, it can be concluded from the obtained data that a marker system of six SSR markers is effective for the differentiation of 21 studied maize lines.

However, it should be noted that comparing the results of cluster analysis of maize lines by means of protein markers and DNA markers, the difference between clusters was noted. It was determined that genetically close lines according to the spectra of storage proteins have a significant genetic distance according to SSR markers. Thus, the closest lines by the protein markers DK272 and DK273 were sufficiently distant from SSR markers, genetic distances value was 2.83. The same situation was observed with another pair DK742 and DK325 lines, which were close in zein spectra, with genetic distances for SSR markers 2.45.

To determine the relationship between genetic distances for protein and DNA markers, Mantel correlation analysis was performed (linear correlation Pearson) (Fig. 5).

As a result of the analysis, measures of calculated significance level p-value and the correlation coefficient r (AB) for the theoretical significance level α=0.05 were determined, which, according to the interpretation of the test, allowed to accept one of the analysis hypotheses about the presence (Ha) or the absence of correlation (H0).

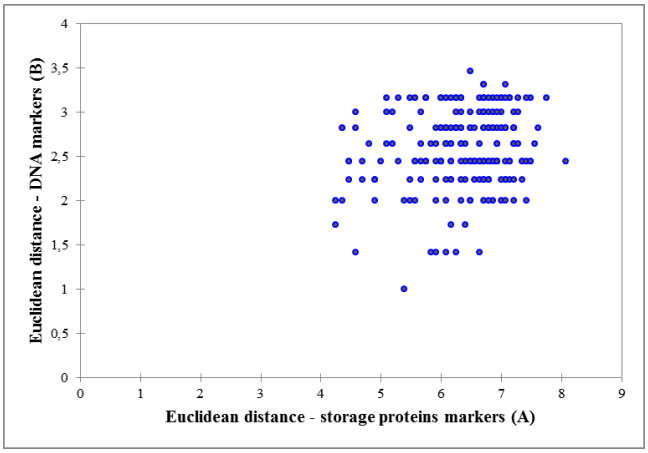

Fig. 5. Relationship between genetic distances of the maize lines according to protein and DNA markers

It is known that the hypothesis H0 about the absence of correlation is accepted on the assumption that $p>\alpha$. As a result of our research, calculated p-value (0.009) was lower than the significance level $\alpha=0.05$, therefore, it is necessary to accept alternative hypothesis Ha about the presence of correlation (Diniz-Filho *et al.*, 2013). The correlation coefficient r(AB) is 0.18. Consequently, as a result of the analysis, the presence of a weak correlation between marker systems for the determination of the maize lines' polymorphism according to the spectra of storage proteins and DNA markers was determined.

It is known that zeins are encoded by families of closely related structural genes located in two chromosomes of maize (Soave *et al.*, 1981; Soave and Salamini, 1982). Based on individual clones hybridization with genomic DNA, it has been proven that zeins are coded by 150 genes (Zayakina *et al.*, 1998), which are located on chromosomes 4 and 7 (Wilson *et al.*, 1989). Based on the study of linkage between zein coding genes by applying isoelectric point electrophoresis of zeins in polyacrylamide gel and isoelectric point electrophoresis in agarose gel. Two clusters are located on a short shoulder of 4th chromosomes and one cluster on 7th chromosomes.

According to data presented by Smith *et al.* (1997) microsatellites, which we used in our work, are localized on different chromosomes. In particular, it is reported that microsatellite reiterations phi079 and phi034 are located on chromosomes 4 and 7, respectively. However, it should also be noted that other markers are localized on distinct chromosomes: phi073 - on chromosomes 3, phi085 on 5, phi022 on 9 and phi062 on 10 chromosomes. Considering that only microsatellite markers phi079 and phi034 have the same spatial localization with zein encoding genes, it can be assumed that this fact is due to the presence of a weak correlation between marker systems.

It is known that the distribution of maize maturity groups according to FAO is based on the estimation of the leaves number per plant, the length of the vegetation period and the sum of effective temperatures (Andriuscenko and Kryvyckyi, 2007; Bavec and Bavec, 2002). Excluding the component of the regional placement (the sum of effective temperatures), characteristics such as the number of leaves and the length of the vegetation period are genetically determined. As a result of the research, it has been determined that there is a moderate correlation between the FAO indexes of the studied lines and the presence of zein components with different electrophoretic mobility. It has been established that the component of zein with Rf 50 is a characteristic for the maize lines of middle-early, mid-season, and middle-late maturity groups (FAO>250), the correlation coefficient is 0.44. The correlation between the presence of a component with Rf 47 in maize lines, FAO of which was less than 230, i.e. in the middle-early and mid-season lines (correlation coefficient 0.48), was also determined. For components with Rf 34 and Rf 57 inverted correlation with FAO indicator was estimated. Consequently, the correlation coefficient -0.68 shows that lines with FAO less than 230 (middle-early and mid-season groups) the Rf 34 component is absent. Its presence is noted only in the middle-early and middle-late lines. The presence of a component with Rf 57

is noted for lines with FAO 190-210 and 280-320 (correlation coefficient -0.44). Sidorova *et al.* (2012) obtained similar data, according to the results of research the component with Rf 57 was identified in early-season maize groups. Thus, it has been established that a positive correlation between FAO and zein components with particular electrophoretic mobility indicates that with increasing of FAO the frequency of the corresponding component in particular mature group of maize increases.

The conducted studies allowed revealing correlation on the level of 95% between FAO index of the maize lines and presence of particular allele for several studied SSR markers. Consequently, moderate correlation between FAO indexes and identified alleles with phi034 marker was determined. It was found that the presence of allele with a size 138 bp is distinctive for the early-season and middle-early maturity group, and 142 bp allele is identified predominantly in the middle-early, mid-season and middle-late groups. The correlation coefficient was 0.45. The phi085 marker has a moderate inverse correlation (correlation coefficient -0.32), which shows that with increasing of 230 bp allele frequency, the FAO index decreases. That is noted that the allele of the specified size is distinctive for the overwhelming majority for lines with FAO<220, which belong to the middle-early and early-season groups. Weak correlation was identified for phi062, phi079 and phi022 markers, the correlation coefficients were 0.20, 0.18 and 0.17, respectively. No correlation dependencies were discovered for the phi073 marker. This indicates the lack of regularities of the particular allele's presence according to these markers and the belonging of the studied lines to the particular maturity groups.

It should be noted that the mechanism of leaves' initiation and the duration of the vegetation period have a polygenic structure of the coding and regulating genes, the effect of which is related to the activity of auxins and cytokinins (Wang *et al.*, 1999; Sinha, 1999; Werner *et al.*, 2001; Juarez *et al.*, 2004; Ezhova and Vu, 2008; Alter *et al.*, 2016; Li *et al.*, 2016). On this basis, without further research, it is not possible to determine the nature of the correlation between FAO indexes and molecular genetic markers. However, the presence of such dependencies allows indirectly predict a group of lines' maturity or obtained on its basis hybrid combinations, based on particular identified zein components or alleles, which were identified by the investigated DNA markers. Thus, obtained results show the effectiveness of using molecular genetic markers for the evaluation not only the genetic diversity of maize lines, but also their application in comprehensive assessment of the economic characteristics of lines and selection material.

CONCLUSIONS

As a result of the studies, polymorphism of 21 of the maize lines according to the DNA markers and seeds' storage proteins was determined. It was identified that the most similar according to zein spectra were DK742 and DK325, DK272 and DK273 lines, genetic distances between them was 4.24. It is noted that the most similar lines differed by at least five components. The correlation between zein components with different electrophoretic mobility was determined, which

indirectly confirms the complex organization of zein coding genes. It was shown that the distribution of lines according to SSR markers differed from the distribution obtained by electrophoretic spectra of zein. It was found that the most similar lines according to SSR markers were lines DK680 and DK633/266, genetic distances between them was 1.0. The polymorphism level of the investigated marker system varied from 0.18 (phi062) to 0.83 (phi085) and averaged on the level 0.59. It was determined that there was correlation between two marker systems. The obtained correlation coefficient (r(AB)=0.18) indicates a weak correlation between the genetic distances of the studied lines according to protein and SSR markers. Correlation relationship between FAO indexes of the studied lines and presence of particular zein components or alleles according to SSR markers have been determined. Obtained data regarding usage of molecular genetic markers indicates the possibility of their usage for the maize lines evaluation in order to determine the most favorable combinations for breeding. Correlation bonds, which were identified for studied parameters, can be used in breeding work to predict the future characteristics of the obtained lines and hybrids.

ACKNOWLEDGEMENT

We would like to thank the staff of the Institute of grain crops of NAAS (Dnipro) for providing research materials and effective cooperation.

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Original Scientific paper
10.7251/AGRENG1903018L
UDC 634.11

THE IMPACT OF LIGHT PENETRATION INTO CANOPY AND SEASONALITY ON PHOTOSYNTHETIC INDICES IN APPLE TREE LEAVES

Kristina LAUŽIKĖ[1]*, Giedrė SAMUOLIENĖ[1,2], Nobertas USELIS[1]

[1]Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry, Kauno Str. 30, LT-54333 Babtai, Kaunas dist., Lithuania
[2]Vytautas Magnus University Agriculture Academy, Studentų str. 11, LT-53361 Akademija, Kauno r.
*Corresponding author: k.lauzike@lsdi.lt

ABSTRACT

The aim of this study was to analyse the impact of light penetration into canopy and the effect of distances between technological tools and seasonality on photosynthetic behaviour. Apple tree cultivar 'Auksis' was grafted onto super-dwarfing rootstock P22 and planted at different distances (from 0,25 m to 1 m in rows, while space between rows was 3 m). Photochemical reflectance and plant senescence reflectance indices were measured at two heights: 1.0 – 1.2 m above ground and 1.8 – 2.0 m above ground; specific leaf area, fresh and dry weight were evaluated from all the canopy. Strong positive correlations were determined between photochemical reflectance index and plant senescence reflectance index in higher and lower levels of the canopy. Strong negative correlations were determined between photochemical reflectance index and plant senescence reflectance index and between specific leaf area and dry and fresh mass ratio. Increasing density between apple trees from 1 m to 0.5 m led to increase in photochemical reflectance index and specific leaf area, but plant senescence reflectance index decreased. Meanwhile, seasonality had significant impact on specific leaf area formation and dry to fresh weight ratio. Dry and fresh weight ratio increased by 5% in autumn compared to summer. Our results indicated that with decreased light penetration into canopy photochemical reflectance index decreased, but plant senescence reflectance index increased. Moreover, in autumn, trees prepare for winter by storing more nutrients and leaves accumulate more dry mass.

Key words: *apple tree, seasonality, light penetration, planting density.*

INTRODUCTION

In regard to increasing global food demand, horticulture poses new challenges to grow large quantities of good quality fruits in small areas. Fruit yield depends on photosynthetic processes ant it is important to optimize photosynthetic productivity. The main part of the biomass quantity is dependent on the optimal

photosynthesis system work (Long et al., 2006, Hüner et al., 2016). Photosynthesis is close not only to individual leaf but also on the light penetration through the canopy (Song et al., 2013). Young trees cover little with each other, but as the canopy is formed, the amount of light penetration into the canopy on the tree decreases (Cherbiy-Hoffmann et al., 2012). However, high density planting principle is to make the best use of space and light by planting of a greater number of plants through manipulation of tree size to get optimal return from tree (Choudhary et al., 2015). High – density planting can enhance the productivity of apple fruits, however, there must be right tree architecture for higher light interception, water and nutrition accumulation (Sharma and Jaipaul, 2014, Liu et al., 2016; Zhang et al., 2017). Variance of carotenoids content and their proportion to chlorophylls are therefore commonly used for the analysis of plant physiological state. Photochemical reflectance index (PRI) and plant senescing reflectance index (PSRI) are based on carotenoids and chlorophylls and are typically used to characterize the changes of physiological status of vegetation. Thus PRI characterizes the photosynthetic efficiency, the plant senescing reflectance index (PSRI) was found to be sensitive to the carotenoids and chlorophyll ratio and was used as a quantitative measure of leaf senescence (Merzlyak et al. 1999, Sims and Gamon 2002, Garbulsky et al. 2011). Specific leaf area (SLA) is calculated as leaf area per unit mass. Konopka et al. (2016) found that SLA was the smallest at the top of the canopy in full light conditions and increases with shading. Larger SLA with increasing shading is likely an adaptation for more efficient light interception in low light conditions (Niinemets et al., 2001). The main aim of this study was to analyse the impact of light penetration into canopy and the effect of distances between technological tools and seasonality on photosynthetic behaviour.

MATERIAL AND METHODS

A field experiment was carried out in an intensive orchard at the Institute of Horticulture, Lithuanian Research Centre for Agriculture and Forestry, Lithuania. The apple tree (*Malus domestica* Borkh.) cultivar 'Auksis' was grafted on super-dwarfing rootstocks P22. Trees were planted in distances: 0,25 m, 0,5 m, 0,75 m and 1 m between trees in rows, while space between rows was 3 m. Pest and disease management was carried out according to the integrated plant protection practices, the orchard was not irrigated. Soil conditions of the experimental orchard were as follows: clay loam, pH 7.3, humus 2.8%, P_2O_5 255 mg kg^{-1}, K_2O 230 mg kg^{-1}. Three single trees were selected randomly. Measurements and leaf samples were taken in the middle of July (BBCH 73 – 75) and at the end of August (harvest time BBCH 87-88).

Photochemical reflectance index (PRI)was evaluated using non-destructive method (CI-710 Leaf spectrometer, CID Bio-Science, WA USA) from five leaves from each tree at two heights: 0.8 m above ground inside the canopy and 1.5 m above ground outside the canopy. The PRI combines reflectance at 531 nm (R531) with a reference wavelength insensitive to short-term changes in light energy conversion efficiency (R570) and normalizes it:

PRI= $(R_{531} - R_{570})/ (R_{531} - R_{570})$

Nitrogen balance index (NBI) was evaluated using non-destructive measurements of leaf chlorophyll and flavonoid content in the epidermis (Dualex ®4, Dynamax Inc., USAfrom five leaves from each tree at two heights: 0.8 m above ground inside the canopy and 1.5 m above ground outside the canopy.

To determine the leaf area (cm^2), twenty leaves were randomly sampled from the whole tree canopy and measured with a leaf area meter (AT Delta – T Device, Burwell Cambridge UK). The dry mass of twenty leaves was determined by drying apple leaves at 70ºC (Venticell 222, Medcenter Einrichtungen, Gräfeling, Germany) to constant weight (48 hours). SLA was defined as the leaf area per unit of dry leaf mass, usually expressed in $cm^2 g^{-1}$.

The data were processed using two-way and three-way analysis of variance (ANOVA) at the confidence levels $P \leq 0.05$ and $P \leq 0.01$.

RESULTS AND DISCUSSION

Plant senescence reflectance index (PSRI) significantly changed during the season. The trees were less stressed on the beginning of July, especially on largest distance on the top of canopy (Fig. 1). As Merzlyak et al. (1999) determined that the PSRI goes less than 0 it is the begging of leaves senescence. The senescence of the most densely planted trees begun from the beginning of apple maturity (BBCH 73 – 75), meanwhile all other planting densities resulted the senescence processes only during the harvest time.

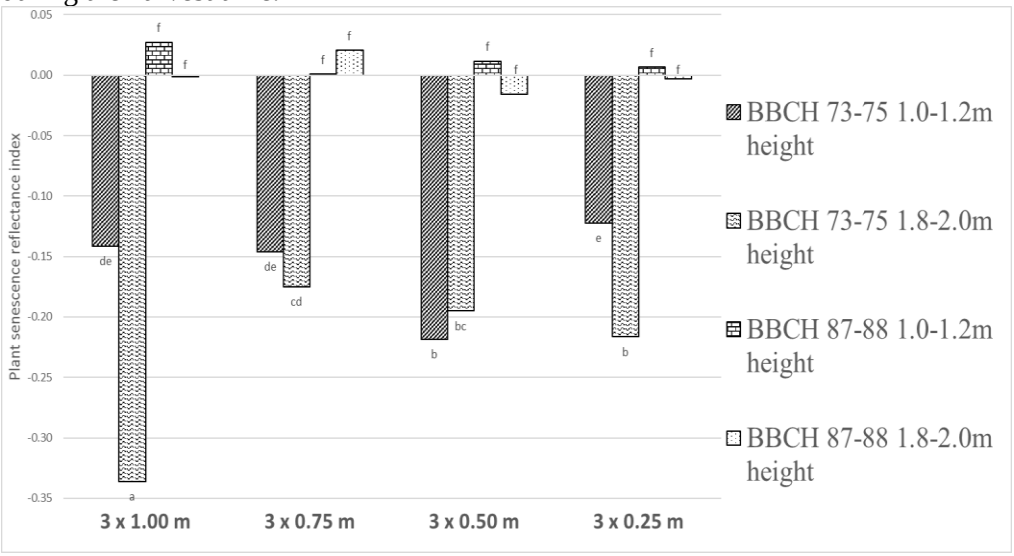

Figure 1 Plant senescence reflectance index in two heights in apple trees. Averages followed by different letter between treatments indicate significant differences according to the Duncan's least significant difference test ($P < 0.05$).

No significant impact for PRI was determined at the begging of apple maturity. Weng at al. (2010) found that PRI decreased in mango tree leaves with the

increased illumination. Meanwhile, increased apple trees density from 3 x 1.00 m to 3 x 0,75 m PRI also increased 1.5 – 2.0 times irrespective of any further increase in density (Fig. 2). PRI can serve as an indicator of the seasonal variation of potential PSII efficiency (Weng et al. 2006). The leaves were rapidly senescing, as the ratio of chlorophylls to carotenoids decreased in the autumn. Because of that, PRI decreased up to 3 times on harvest time compared to the beginning of apple maturity (BBCH 73 – 75).

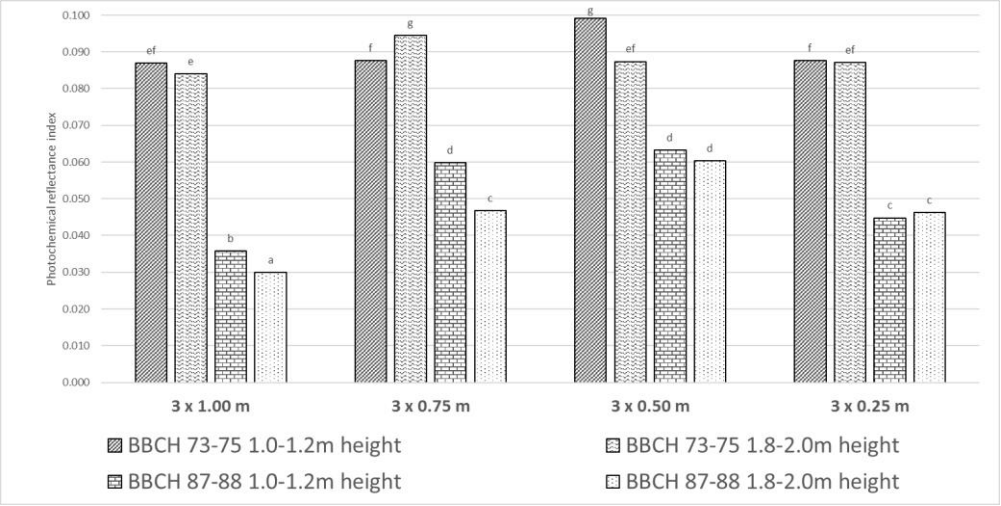

Figure 2. The effect of light penetration into the canopy, the distance between trees and seasonality on the photochemical reflectance index. Averages followed by different letter within between treatments indicate significant differences according to the Duncan's least significant difference test ($P < 0.05$).

SLA was significantly higher at the beginning of apple maturity (BBCH 73 – 75), leaves accumulate less dry matter compared to harvest time. Jagodzinski et al. (2016) shows diferences between flowering ang growing stages during different seasons in 12 forest herb species, and the trends are the same as in our research. Decreased density between apple trees resulted the increase of SLA (Fig. 3). This means that there is bigger competitive stress between apple trees and they form bigger leaves, but less dry matter. Bigger leaves is response to lack of light (Niinemets et al., 2001). Higher SLA can lead to higher photosynthetic efficiency (Wright et al., 2004), but also shows that leaves were shaded and indicates ligth deficiency (Wyka et al. 2012, Neufeld and Young, 2014, Konopka et al. 2016). Apple tree leaves accumulated more dry matter during harvest time compared to summer time. The higher dry to fresh weight ratio was obtained in leaves from trees planted in distance of 0.75 m between apple trees. By the increased density between apple trees, the dry mass decreased (at the same time Dry/fresh weith ratio decrease was observed), but it resulted the increase of SLA. This is in agreement with results of Sims et al. (1994), and Poorter & Nagel (2000).

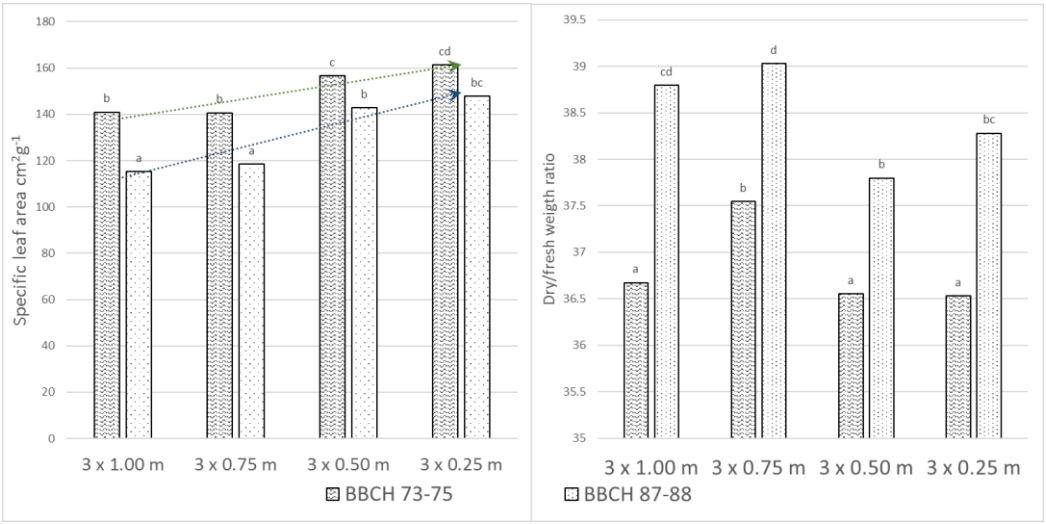

Figure 3 The effect of the distance between trees and seasonality on Specific leaf area and on dry and fresh weight ratio in 'Auksis' apple tree. Averages followed by different letter within between treatments indicate significant differences according to the Duncan's least significant difference test ($P < 0.05$).

CONCLUSIONS

Decreased light penetration into canopy resulted the decrease of PRI, but PSRI increased, the same tendency of photochemical indices variation during both measurements was observed. Increased density between apple trees lead to increased SLA, but it resulted the decrease of dry/fresh weight ratio, however, bigger leaves, but less dry weight were formed. The accumulation of dry weight was more intensive in autumn.

ACKNOWLEDMENTS

This work was carried out within the framework of the long-term research program 'Horticulture: agrobiological basics and technologies' implemented by the Lithuanian Research Centre for Agriculture and Forestry.

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Original Scientific paper
10.7251/AGRENG1903025K
UDC 634.75

ABSCISIC ACID AND IRRIGATION LEVELS EFFECTS ON MORPHOLOGICAL CHARACTERISTICS OF STRAWBERRY

Burçak KAPUR[1*], Eser CELIKTOPUZ[1], Mehmet Ali SARIDAS[2], Abdul Qaiyom SARWARI[1], Sevgi Paydaş KARGI[2]

[1]University of Cukurova, Faculty of Agriculture, Department of Agricultural Structures and Irrigation, Adana, Turkey
[2]University of Cukurova, Faculty of Agriculture, Department of Horticulture Science, Adana, Turkey
*Corresponding author: bkapur@cu.edu.tr

ABSTRACT

The high value of strawberries creates potential for high rates of employment and farm income in Turkey. Optimizing water application and effective cultivation practices are of considerable importance in improving strawberry yield. In this study, the effects of four different irrigation regimes and Abscisic Acid application (ABA use and control) effects on the leaf area, plant dry matter and crown number of strawberry (*Fragaria* × *ananassa* cv. Rubygem) were evaluated under Spanish type high tunnels conditions. ABA was applied three times starting from March to May via foliar application as 20 μmol L^{-1}. From the initiation of the treatment to the end of the trial, a total of 552, 447, 342 and 237 mm of water were applied to treatments IR125, IR100, IR75 and IR50 respectively. The IR50 treatment caused a significant decline in morphological parameters, indicating that the amount of irrigation water did not meet the plant water requirement. The increased amount of irrigation water increased the leaf area, dry matter and the crown number significantly. Furthermore, the ABA application increased the leaf area by 15%, the plant dry matter 12% and crown number by 8%. Under water stress conditions (IR50), ABA significantly increased growth rate as well as increasing leaf area, plant dry matter and the crown number by 13%, 12% and 11%, respectively, when compared to the control plot. Consequently, in the protected cultivation, the IR125 irrigation level and the ABA application enhanced vegetative growth and in turn the total marketable fruit yield and its components.

Keywords: *Leaf area, Dry matter, Class A pan, High tunnel, Rubygem.*

INTRODUCTION

Water scarcity depresses the crop production that occurs a major constraint in providing the food demand worldwide. Approximately 45% of the world agricultural areas are exposed to incessant water shortage, where 38% of the world population lives (Bot et al., 2000). Nowadays, almost 18% of the global farmland

is under irrigation as well as up to 40% of the global food supply is produced from this area (Somerville and Briscoe, 2001; Hussain *et al*., 2012). Therefore, to enhance the efficiency of water in irrigated agriculture, optimal strategies should be developed to avoid the risk of future water supply shortages. Thus, advanced crop drought resistance applications and using optimal irrigation scheduling appears as a significant concern in agricultural production, mainly on water-sensitive crops. Even though it's high sensitivity to water stress, strawberry has a commercial value in Turkey due to the raised market demand. Turkey is the leading country in the strawberry production of Europe by 400.167 metric tons in 2017 (TUIK, 2019). In this regard, water stress research and the investigation of various agricultural practices have gained popularity in the study of strawberries. Limited irrigation is generally associated with the reduction of morphological parameters and thus negatively affects the strawberry yield (Liu et al., 2007; Giné Bordonaba and Terry, 2010).

In this context, the crucial object in agricultural applications and research is how to struggle the water stress, within the environmentally and economically sustainable procedures. Although the extensive use of irrigation in strawberries, their specific water requirements are uncertain (Lozano *et al*., 2016). In the earlier studies, a wide range of irrigation water applications have been reported, but they differ depending on the cultivar, production method, climate and water requirement calculations (Hancock, 1999; Lozano *et al*., 2016). In this way, variations in irrigation water use suggest that locally conducted trials are required to develop the irrigation management in specific regions and cultural systems (Kirschbaum *et al*., 2004). Furthermore, exogenous applications to crops are vital to improve the yield and quality under stress conditions. Abscisic acid (ABA) is one of the major exogenous applicants, which is a plant growth regulator and an osmotic protector that increases the degree of tolerance of the plants against water stress (Heschel and Hausmann, 2001; Wang *et al*., 2003). Plant physiological processes, growth, development, productivity, and responses to abiotic stresses are also affected by ABA applications. Moreover, ABA application effects were remarkable in terms of some morpho-physiological parameters under water stress conditions (Hussain *et al*., 2012). The effect of ABA on growth responses of strawberry under drought stress conditions has not yet been well studied. Therefore, in this sense, this trial is focused on the effect of foliar spray of ABA on plant morphological parameters of strawberry which induces the yield under various irrigation regimes.

MATERIALS AND METHODS

The experiment was executed inside the high tunnel at the Cukurova University experimental farm (latitude: 36^{o} 59`N, longitude $35^{o}$ 27`E, 20 m above sea level). A typical Mediterranean climate prevails in the experimental area, with cool, rainy winters and hot, dry summers. The soils at the site have been classified as Xerofluvents of the Entisol order with heavy clay texture. The bulk density for the top 0.3 m is 1.6 g cm^{-1} and the pH is 7.6. The water content at field capacity and permanent wilting point are 36% and 16%, respectively. The strawberry (*Fragaria*

× *ananassa* Duch.) cultivar 'Rubygem', of short day type, earliness, good taste and aroma, was planted on September 22 (referred to as 0 days after planting (DAP)) 2017 and cropping continued until June 11, 2018. The frigo plant material was used. The high tunnel was made of a steel frame covered by 0.1 mm thick transparent polyethylene (PE) film, with a center height at 2.50 m and 0.8 m at the open sides (40 m long and 6.5 m wide). To monitor temperature and humidity, climate station was placed in the center of the high tunnel 2 m above soil surface. The area inside the tunnel was heated solely by solar radiation.

The strawberries were planted in trapezoidal raised beds measuring 0.70 m at the base, 0.50 m at the top, with a height of 0.30 m, and a 0.30 m distance between each bed. Each were covered with a 0.05 mm thick, two-sided polyethylene mulch cover, having a grey upper side and black under side, (in accordance with conventional cultural practices in the area) with surface drip irrigation installed down the center. Strawberries were planted in two rows, 0.3 m apart, with plants set 30 cm apart, to an equivalent plant density of 6.65 plants m^{-2}. Each tunnel had four beds. After planting, sufficient water was applied until the plants were well developed. Fertilizer was applied uniformly to each treatment by drip irrigation and foliar application of agricultural pesticides served to control foliar and fruit diseases.

The trial was implemented as a 4×2 factorial scheme of irrigation levels and Abscisic Acid use, in a split-plot design with 4 replicates (blocks) combined over six periods, totaling 32 plots. Applications (ABA use and control) were designed over the main plot and different irrigation regimes were arranged as the sub plots. Furthermore, approximately six months after planting, three times starting from March to May via foliar application, 20 μmol L^{-1} Absisic Acid was applied (March 07, April 05, May 08, 2018). The four irrigation treatments were designated IR50, IR75, IR100, and IR125, where the water quantities applied were 0.5, 0.75, 1.00 and 1.25 times of the pan evaporation (Epan). Epan value was determined using the US Weather Service Class A pan, with a standard 120.7 cm diameter and 25 cm depth, and placed over the crop canopy in the center of the high tunnel. Four irrigation treatments were established in four beds of four, 10 m by 4 m plots, with 266 plants per plot. The irrigation amount was calculated as reflected at Kapur et. al. (2018). From the initiation of the treatment to the end of the trial, a total of 552, 447, 342 and 237 mm of water were applied to treatments IR125, IR100, IR75 and IR50 respectively.

In order to evaluate the morphological responses of strawberry, the samples were taken in May which is the active harvest period. Evaluation of the leaf area (LA), above ground dry matter (DM), crown number (CN), was conducted to characterize the vegetative growth of strawberry under various applications. Leaf area was measured with a leaf area meter (model 3050A; Li-Cor Lincoln, NE, USA). To obtain a value for the dry matter of the crop, the above-ground tissue was dried in an oven at 70 °C until the dry-weight was maintained. The same plants were used to determine the crown number. The obtained data were analyzed with the statistical program JMP version 5.0.1 (SAS Institute Inc., Cary, NC, USA).

ANOVA was calculated to determine the effects of irrigation regime and bio-stimulant on the observed parameters, combined over six periods. A Least Significant Difference test was performed to examine the differences among groups. Comparisons that yielded $P \leq 0.05$ were considered statistically significant. Additionally, using JMP 5.0.1., the multivariate method was used to determine the correlation among all the obtained results, with $P \leq 0.05$.

RESULTS AND DISCUSSION

The responses of strawberry plants to different treatments are presented in Table 1. The measured parameters, LA, DM and CN, ranked similarly. The IR50 treatment caused a significant decline in morphological parameters, indicating that the irrigation amount did not meet the plant water requirement. The higher amounts of irrigation water produced plants with more leaf area and dry matter development. Diminishing growth rate is one of the earliest responses of plants to water deficit. Similarly, reductions in LA and DM have been noted under water stress conditions previously (Liu *et al*., 2007; Grant *et al*., 2010; Grant *et al*., 2012; Ghaderi *et al*., 2015). According to our results, 75 cm^2 LA, 0.06 CN and 0.7 g DM increased per plant for a water level of about 10 mm rise. Leaf area enhancement assesses light interception and is a major parameter in determining plant productivity (Gifford *et al*., 1984; Koester *et al*., 2014). Therefore increased leaf area increased the yield of strawberry in this study (yield data were not shown). Our results are in accordance with that of Yuan et al. (2004), that plant leaves and above-ground biomass with total berry yields all increased when the amount of irrigation water increase.

The effect of the ABA was significant in LA, while CN and DM were not significantly increased. The average values of the parameters increased by 15%, 12% and 8% for LA, DM and CN respectively. The irrigation level and ABA interaction insignificantly affected the LA DM and CN. Under water stress conditions (IR50), ABA significantly increased growth rate as well as increasing LA, CN and DM by 13%, 11% and 12%, respectively, when compared to the control plot. Previous works have found that exogenous applicants both promote plant growth and enhance abiotic stress tolerance (Battacharyya *et al*., 2015). Similar to our results, application of the salicylic acid resulted in increasing the leaf number in strawberry under water stress conditions and drought stress reduced dry matter in strawberry cultivars. Under water limited conditions, application of the salicylic acid increased dry matter, irrespective of the cultivar (Ghaderi *et al*., 2015). Moreover, ABA is known to play an important role in enhancing plant water use efficiency under environmental stress (Jamalian *et al*, 2013). Therefore, ABA is recommended to act as a suitable solute that manages the osmotic potential in the cells (Arshi *et al*., 2005; Caballero *et al*., 2005; Bartels and Sunkar, 2006) and considered to undertake a major role in the protection mechanisms of stressed cells. According to Serraj and Sinclair (2002), osmotic adjustment is the main physiological response to maintain the growth of crops under water stress conditions. Furthermore, the growth and development of vegetation is controlled by phytohormones, like ABA, and gibberellic acid that effect plant growth by

regulating the growth activity, thus explaining the improved growth of strawberry determined in the present study (Khan *et al*., 2009). It is possible that exogenous application increased the leaf area, and improved light interception, thereby heightening the photosynthetic rate and increasing plant productivity (Koester *et al*., 2014). However, aforetime, the cost of producing ABA was too high to support its application as a plant growth regulator, but in these days ABA production methods have been improved and application in cash crops could be advised (Cantin *et al*., 2007; Ferrara *et al*., 2013). The irrigation and ABA application interactions do not significantly differ, probably due to a major variability in the applications. LA, CN and DM change between 1737-4351 cm^2, 3-5.3 and 45.3-72.2 g, respectively.

Table 1. Effects of irrigation regimes and ABA on morphological parameters of strawberry

Leaf Area (cm^2)					
Application	Irrigation Regime				Ave. App.
	IR50	IR75	IR100	IR125	
Control	1737	2710	3603	4088	3035 B
ABA	1956	3658	3960	4351	3481 A
Ave. Irrigation	1846C	3184B	3781AB	4220A	
LSDirr***=624.3		LSDapp**=441.4		LSDirrxapp= N. S.	
Crown Number					
Application					Ave. App.
	IR50	IR75	IR100	IR125	
Control	3.0	3.6	4.6	5.0	4.0
ABA	3.3	4.0	4.3	5.3	4.3
Ave. Irrigation	3.2C	3.8BC	4.5AB	5.2A	
LSDirr***=0.78		LSDapp= N. S.		LSDirrxapp= N. S.	
Dry Matter (g)					
Application					Ave. App.
	IR50	IR75	IR100	IR125	
Control	45.3	48.7	63.7	64.9	55.7
ABA	50.9	55.8	70.0	72.2	62.2
Ave. Irrigation	48.1B	52.2B	66.9A	68.6A	
LSDirr***= 11.8		LSDapp= N.S.		LSDirrxapp= N. S.	

[1]Differences between the means were showed with different letters.
[2] N. S.: Not Significant, ***: $p < 0.01$; **: $p < 0.05$.

CONCLUSIONS

The appropriate and efficient use of drip irrigation systems is significant regarding reduced growth and yield both caused by excess and inadequate irrigation bound to water stress. The effects of different irrigation water applications based on the Class A pan evaporation on strawberry growth were studied in a high tunnel drip irrigation experiment. The leaf area, crown number, dry matter all increased with the increasing amount of irrigation water from IR50 to IR125. The optimal amount of irrigation water is about 552 mm and the optimal crop pan factor is about 1.25

for strawberry growth inside the high tunnel under the Mediterranean environment conditions. Applying water by drip irrigation in relation to the amount of water evaporated from a standard Class A pan is a suitable, simple and low cost method. Thus, strawberries grown inside the high tunnel could be irrigated using a pan factor of 1.25 as a guideline for irrigation during the full vegetation period. Furthermore, the ability of the exogenous compatible solutes, such as ABA, to counteract the water stress effects in strawberry (*Fragaria* × *ananassa* Duch. cv. Rubygem) was investigated. However, the economic evaluation of the applications cost is important for the final decision.

ACKNOWLEDGEMENTS

This work was financially supported by the Coordination Unit of the Scientific Research Projects of the Çukurova University via the project FLY-2018-11307.

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Original scientific paper
10.7251/AGRENG1903033B
UDC 338.43.02(675.97)

THE CONTRIBUTION OF MICROFINANCE TO THE RESILIENCE STRATEGIES OF SMALLHOLDER TEA FARMERS IN BURUNDI

Pierre Claver BITAMA[1], Philippe LEBAILLY[1], Patrice NDIMANYA[2], Emery Gaspard SABUHUNGU[2], Philippe BURNY[1]

[1]Economy and Rural Development Unit, University of Liege-Gembloux AGRO-BIO-TECH, Belgium
[2]Rural Economy Unit, University of Burundi, Burundi
*Corresponding author: pierreclaverbitama@yahoo.fr

ABSTRACT

Microfinance services are of undeniable importance in the development of agriculture and in improving living standards in rural areas. However, their accessibility in developing countries is problematic. The objective of this article is to assess the contribution of microfinance to improve the living conditions of the population in rural areas of Burundi. An exploratory survey was conducted among 120 smallholder tea farmers in 2018 in two zones (Ijenda and Teza). These smallholder tea farmers are between 30 and 86 years old with a basic level of education. The results of the survey showed that the loans made it possible to carry out small projects and met some urgent needs. However, the level of indebtedness was low due to lack of collateral guarantee and the interest rate was relatively high. In addition, the information collected in Microfinance Institutions (MFIs) revealed a lack of support services within MFIs to enable optimal allocation of credits. To compensate for financial shortfalls, smallholder tea farmers are developing mechanisms for saving in kind and tontine systems with multiple socio-economic roles built up. Credit beneficiaries in MFIs are increasingly losing interest in the MFIs credit systems in favour of tontines. In Ijenda zone, only 37.5% want to renew the credits against 41.4% in Teza zone.

Key words: *Burundi, microfinance, MFIs, tea, tontines.*

INTRODUCTION

A common feature for rural farmers in developing countries is the lack of resources - natural, human, financial, physical and technological in quality and quantity (Maxwell, 2000; Adjei et al., 2009) and rural farmers with few resources have a lower capacity for resilience to risks and vulnerabilities (Baumann, 2002; Mosley and Rock, 2004). Indeed, there is a close relationship between vulnerability and resource ownership (Moser, 1998) because the lack of resources is the cause and consequence of poverty (World Bank, 2000). Some authors (Khandker, 1998;

Henry & Schimmel, 2011) postulate that rural farmers' access to financial resources is one means by which they can reduce poverty and increase productivity. The authors (Matin et al., 1999; Kessy and Urio, 2006) confirm that microfinance institutions (MFIs) are essential to the extent that they can: (*i*) reduce poverty or improve the standard of living through increased income; (*ii*) increase the capacity of farmers; and (*iii*) develop entrepreneurship's potential. Microfinance is generally considered as a provider of financial services to individuals or groups of individuals who would not have had access to traditional banking services. They offer a variety of products and services such as "micro-credit", "micro-savings", "micro-insurance" and training for the efficient use of financial resources. These services are mainly provided to micro-entrepreneurs, low-income people and the poor in order to reduce and mitigate the risks and vulnerabilities that threaten these groups of individuals (Hulme et al., 2009). In Burundi, poverty reduction is at the heart of the concerns of the State authorities despite countless challenges and constraints in the country. To reduce poverty and revitalize the country's economy, State authorities have undertaken strategic growth and poverty reduction programmes in the participatory process involving local community representatives, civil society, private sector, parliament, central government and development partners for redistributive growth for the poorest (Rufyikiri, 2012). Despite this commitment to improve the well-being of the country's population, there is a small private investment sector, which is the driving force behind economic growth. In line with the implementation of rural development strategies, the State authorities are convinced that microfinance is the driver of development in rural areas. To this end, they focus on the microfinance sector in particular. In addition, a desk study on the banking sector in Burundi revealed the inaccessibility of the rural population to the services of classical banks, despite the fact that they represent 75% of all financial assets. The microfinance sector includes both informal and formal sector stakeholders. The informal sector includes endogenous savings and credit practices and the formal sector includes institutions such as savings and credit institutions, NGOs, non-profit organizations, etc. (Ashcroft et al., 2007). The objective of this article is to assess the importance of microfinance in the resilience strategies of smallholder tea farmers in Burundi.

MATERIALS AND METHODS

In order to achieve the research objective, a survey was conducted among 120 smallholder tea farmers in the Ijenda and Teza areas in January and February 2018. Due to time and resource constraints, we chose two areas near the capital (Bujumbura) of Burundi from among the five tea-growing areas (Rwegura, Teza, Ijenda, Tora and Buhoro) in the Mugamba natural region. Stratified random sampling was used to give all small tea farmers an equal chance to be selected for the entire tea acreage of the two surveyed areas (Marshall, 1996). Qualitative data were gathered using semi-structured questions from these 120 smallholder tea farmers and a few MFI staff members in the two selected areas. Secondary data were also collected to complete our survey. When collecting primary data, we first

highlighted the socio-demographic characteristics (age, level of education, gender, marital status), main activities and sources of income that enable smallholder tea farmers to ensure their survival. Then, we placed particular emphasis on the contribution of microfinance and the relationships it maintains with smallholder tea farmers: savings formation, granting loans, reimbursement conditions, the interest rate applied, repayment deadlines, etc. Data exploitation and interpretation are carried out through content analysis (Patton, 2002; Duriau et al., 2007; Srivastava and Thomson, 2009).

RESULTS AND DISCUSSION

Savings situation of smallholder tea farmers

The results of the survey reveal that the savings of smallholder tea farmers in MFIs is low: 5.2% of the population surveyed in the Ijenda area save in cash and no savings have been found among smallholder tea farmers in Teza. The holding of accounts in MFIs is almost non-existent in the Ijenda area (zero in the Teza area). The investigation also shows that some bank accounts are closed after opening due to a lack of liquidity to fund them. The survival of smallholder tea farmers is based on income diversification (sale of green tea leaves, food products, livestock, etc.). By default of savings in MFIs, smallholder tea farmers develop other forms of savings. Thus, two types of savings were identified. On the one hand, income that is not allocated in the purchase of consumer goods or inputs is saved in the purchase of domestic animals to obtain organic manure or for subsequent sale if cash if needed. On the other hand, smallholder tea farmers save money in the form of tontines. The latter are organized in the form of small associations of 5 to 30 persons. Members of these small tontine associations contribute small amounts, ranging from 500 to 2500 Burundian franc (BIF) (1.00 € = 2070.69 BIF on July 8th, 2019) per week. Contributions can also be made in kind: in the association "Tugwize uburimyi" for example, the associates save 10 kg of green tea leaves per week. These small amounts saved are redistributed among members after one year. In the case of non-distribution, the members of these small associations carry out small projects such as the breeding of domestic animals or the rental of land to grow food crops. In addition to these economic benefits, tontines also provide a social role. In the event of risk (death, illness, etc.), they entitle the partners to a small loan at an interest rate of 10%. In addition, smallholder tea farmers have highlighted the importance of tontine associations as a means of learning and sharing information on agricultural and non-agricultural activities. According to them, tontine associations enable them to acquire knowledge about new technologies and agricultural methods that help them to cope with constraints and vulnerabilities. Tontine associations are considered as open-minded.

Known for a very long time (in Europe and Japan), the tontine system is a form of informal mutual aid economy popularized in developing countries. In various forms, the World Bank is very interested in tontine phenomena: it talks about them in Cameroon, Niger, Mozambique, India, Philippines, Indonesia, Bolivia, Mexico, etc. (Lelart, 1990). The role of tontines is crucial among small rural farmers in

African countries and plays an economic and socio-cultural role. Tontines make it possible to carry out a joint project. Through tontines, rural farmers can save funds either for a short or medium-term investment or for a planned and/or unforeseen event, collectively or individually in a context of extreme material poverty. On the social level, tontines encourage the exchange of ideas and mutual support in times of joy or difficulty (Ependa, 2002).

Inaccessibility to microcredits

In the agricultural sector, the financing of some activities through the use of debt is a condition. To carry out certain projects (purchase of arable land, construction of a house, purchase of cattle, etc.), smallholder tea farmers take out loans in MFIs. However, the amounts of credits are very small to carry out a project in the medium and long term. Over a five-year period, our survey revealed that the smallholder tea farmers in the sample contracted credits for a total of 5,850,000 BIF (Table 1). The amounts of each credit are between 100,000 BIF (€ 48.29) and 300,000 BIF (€ 144.88).

Table 1. Number and amount (BIF) of credits (2014-2018) granted to surveyed smallholder tea farmers

	Teza zone		**Ijenda zone**		
Origin of the credit	Number of credits	Amount	Number of credits	Amount	Total amount
COOPEC	12	1,700,000	10	1,450,000	3,150,000
MUTEC	-	-	23	2,250,000	2,250,000
DIFO	3	450,000	-	-	450,000
Total	15	2,150,000	33	3,700,000	**5,850,000 (€2825.4)**

COOPEC- Coopérative d'Epargne et de Crédit (Savings and Credit Cooperative) ; MUTEC- Mutuelle d'épargne et de crédit (Savings and credit mutual societies) ; DIFO- Development Interpeople Finance Operations.
Source: our survey, 2018

Regardless of the amount of credit, loans in MFIs are subject to collateral guarantees. In the surveyed area, the collateral guarantee is the tea plant with an area of at least ten acres. The reimbursement of the credits is made thanks to the income from the tea plant (withdrawal from the source at the time of payment) or from other sources of income. The interest rate varies between 18% and 19% for loans with a maturity of 2 to 3 years. Bank overdrafts are remunerated at 30%, the amount depending on the quantity of green tea leaves sold. Depending on the size of the credit amounts, credit recipients should associate in groups of 5 to 10 smallholder tea farmers. The arable land available to smallholder tea farmers is not a collateral guarantee since these smallholder tea farmers do not have land certificates or land titles. In addition, since the houses are made of non-durable material, they are not mortgaged. Credits are not allocated for small projects only. In some circumstances, they are contracted to ensure the survival of smallholder

tea farmers. During the lean season, credits are directed towards the purchase of consumer goods or to pay for certain needs that require a disbursement of funds, such as the occurrence of an illness in a household, the payment of school fees, etc. Despite these multiple advantages of microcredits, the survey revealed a lack of interest in them. The mortgage constraint and the difficulty of repayment - the interest rate is relatively high - are the causes of the disinterest. In Ijenda only 37.5% want to renew the credits against 41.4% from Teza.

Microcredits have undeniable advantages. They enable rural farmers to overcome periods of shock or hunger. Access to credit makes it possible to obtain new resources to replace those destroyed by natural disasters (World Bank, 2002). Credits make it possible to deal with exceptional situations that require financial resources without being able to place key survival assets on the market or disinvest in human capital, such as dropping out of school or children not attending school because of a lack of financial resources to afford school fees and materials, for example (Barnes, 1996). The constraint of collateral guarantees, relatively high interest rates, payment terms and small amounts received are shared by rural farmers in modest living conditions in some developing countries (Banerjee and Newman, 1993; Rweyemamu et al., 2003; Kessy and Urio, 2006). Inaccessibility to microcredits is a limitation to take advantage of opportunities that may arise (Bebbington, 1999). The situation of MFIs for smallholder tea farmers in Burundi is very different from that of the Grameen Bank in Bangladesh, a genuine microfinance institution for small groups of rural farmers (World Bank, 1996; Hassan & Renteria-Guerrero, 1997). Microfinance in Burundi offers relatively fewer flexible conditions for smallholder tea farmers to take advantage of the services offered to them. In addition, there are donor interventions in many developing countries for microfinance development such as: U.S. Agency for International Development (USAID), International Fund for Agricultural Development (IFAD), Norwegian Agency for International Development (NORAD), Canadian International Development Agency (CIDA), Swedish International Development Authority (SIDA), etc. (Khandker, 1998).

Microcredit support services

The survey revealed a lack of non-financial support services for credit recipients. Training in MFIs is limited to their staff, mainly in terms of IT tools. MFI services should not be limited to crediting only. Microfinance managers have to be concerned about the impact of these credits on the lives of credit recipients in the medium and long term in the event of a good or misallocation of the financial resources received. Our sample is made up of smallholder tea farmers with a basic level of education. At this level, the concepts of interest rates, payment deadlines and/or amounts to be reimbursed are not known to the beneficiaries of loans. The survey revealed that most of them did not know either the interest rate or the payment due date. In Ijenda, 50% of loan beneficiaries did not know the interest rate applied to the contracted loans and 12% gave incorrect interest rates (lower than the rate required by MFIs). In the Teza area, all borrowers have set the interest

rate between 2% and 3% while the interest rate is between 18% and 19%. It is questionable whether MFIs actually provide clear information about interest rates and/or other information about contracted loans or whether this lack of knowledge is due to their level of education. A support service for credit recipients should be mandatory. MFIs need to make credit lenders understand what the money is to be used for in order to take advantage of the loans requested and anticipate repayment issues. It is an opportunity for them to acquire knowledge related to investment, budgeting, saving, borrowing, etc. Sebstad and Cohen (2003) reaffirm that microcredits are exposed to daily, sometimes unexpected financial needs (accident, unexpected illness, etc.) due to the low livelihoods of rural farmers and consequently a misallocation of microcredits results. In addition, the situation of rural farmers may in some circumstances constitute opportunity costs. A support service for rural farmers should be set up within MFIs.

CONCLUSION

Rural farmers in developing countries are in need of financial resources for the implementation of small projects that can improve their well-being. This article shows that in Burundi, access to the microfinance credit system is subject to restrictive measures. To meet these constraints, smallholder tea farmers are building up tontine systems with a socio-economic role. To mitigate the constraints on access to MFIs services, some measures could be implemented. In order not to limit access to credit only to small tea farmers with plantations of a certain size, the collateral guarantee could be extended to food crops. To this end, the repayment of the credits would be deferred over the post-harvest periods of the country's three cultural seasons. Training for credit recipients would precede the disbursement of credits for a better allocation of funds. It would be essential for the local authorities to be involved to facilitate repayment tasks. A positive productivity impact on rural farmers would undoubtedly be remarkable thanks to the acquisition of inputs in sufficient quantity and quality (improved seed, fertilizers, pesticides, etc.) and the implementation of small joint projects. To avoid insolvency in the event of climatic hazards, the public authorities would guarantee the credits. The purpose of MFIs is to reduce poverty among low-income rural and urban populations. The interest rate of these MFIs should be lower than that of traditional banks. To prevent these MFIs from working at a loss, the public authorities would exert a significant influence, as they did on mineral fertilizers, which are subsidized up to 40% of the total price (MINAGRIE, 2011). In addition, tontine supervision could lead to very positive results. Instead of making weekly savings and redistributing them among members at the end of the year, savings could be extended over the medium term. Thus, smallholder tea farmers would make considerable investments in cooperatives.

ACKNOWLEDGEMENTS

The authors would like to thank the smallholder tea farmers and microfinance staff for agreeing to answer their questions.

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Original Scientific paper
10.7251/AGRENG1903042S
UDC 582.475:632.112(65)

IMPACT OF DROUGHT AND SITE CHARACTERISTICS ON VITALITY AND RADIAL GROWTH OF *CEDRUS ATLANTICA* MANETTI IN THE OUARSENIS MASSIF (ALGERIA)

Mohamed SARMOUM[1*], Rafael NAVARRO-CERRILLO[2], Frédéric GUIBAL[3], Fatiha ABDOUN[4]

[1]Laboratory of Plant Physiology and Out Soil Culture, Faculty of Natural and Life Sciences, Ibn Khaldoun Tiaret University, Algeria
[2]Department of Forest Engineering, Group of Evaluation and Restoration of Agricultural and Forestry Systems, DendrodatLab - University of Córdoba, Spain
[3]Mediterranean Institute of Biodiversity and Marine and Continental Ecology (IMBE), UMR 7263 CNRS-IRD, Aix-Marseille University (AMU), Aix-en-Provence, France
[4]Laboratory of Plant Ecology and Environment, Faculty of Biological Sciences, University of Science and Technology Houari Boumediene (USTHB), Algiers, Algeria
*Corresponding author: sarmoum.med82@gmail.com

ABSTRACT

This work investigates the impact of drought and site characteristics on vitality and radial growth of Atlas cedar (*Cedrus atlantica* Manetti) in Ouarsenis cedar forests (Algeria). The choice of this zone was dictated by the appearance of the phenomenon of decline since the 1980s and the lack of study on this subject. Our hypothesis seeks to understand how climatic factors interacted with site characteristics affected radial growth and vitality of Atlas cedar. We used the dendroecological approach where 09 populations of Atlas cedar distributed on the two cedars of Ouarsenis (Theniet El Had and Ain Antar) and covering a varied range of environmental conditions (substrate, altitude, exposure) were studied. The climatic signal recorded in ring-width series of Atlas cedar trees was investigated by bootstrapped response function over the period 1936-2010. The results show a good agreement between the individual curves and those of mean site chronologies, which reflects the influence of climatic factors on tree radial growth. Atlas cedar is very sensitive to rainfall fluctuations throughout the year. This sensitivity is more pronounced for populations located at low altitude, on steep slopes and on sand stone or marl substrates. The dry years induced a significant radial growth decline and triggered massive tree mortality, particularly in 1983, 1984, 1988, 1994 and 2002. The vitality of the species seems to be conditioned by the frequency of drought years.

Keywords: *Atlas cedar, decline, Algeria, radial growth, drought.*

INTRODUCTION

There is now a broad consensus that climate change has already affected or is affecting the functioning and production of plant cover, including forest stands (Spathelf *et al*., 2014). However, the response of forest species is complex and the impact of changing climate conditions on mortality rates observed during the last decade in several species remains poorly understood (Allen *et al*., 2010). In North Africa, climate has been dominated by drought over recent decades (Touchan *et al.*, 2008). Reduced rainfall during the wet period (October-May) and increased temperatures during the same period would result in an increase in the dryness and summer drought (Dai, 2011). This situation may jeopardize the existence of several species including those that are sensitive to drought (Linares *et al.*, 2011). The Atlas cedar is an endemic species in the highest mountains of Algeria and Morocco where it occupies a very fragmented area (Benabid, 1994). This species fits better in humid and sub-humid bioclimate and is averse to the long and repeated droughts (Ladjal *et al.*, 2007). Currently, this species is experiencing severe deterioration marked by a lack of natural regeneration and massive tree mortality, and the absence of silvicultural practices has worsened the situation (Messaoudene *et al.*, 2013). The phenomenon of Atlas cedar decline was first observed in the 1980s, but has become alarming in recent years (Bentouati et Bariteau, 2006). Although the causes of this problem remain uncertain, the hypothesis of impacts of climate change cannot be excluded (Linares *et al.*, 2011; Kherchouche *et al.*, 2013). Drought impact on vitality and radial growth of Atlas cedar has been recently studied (Linares *et al.*, 2013; Slimani *et al.*, 2014). However, these studies have focused on one site, without taking into account the environmental variability. Our project assessed the impact of drought, in addition to other the environmental variables, on the vitality and the radial growth of Atlas cedar through a retrospective approach using tree rings as a recorder of climate signals. We seek to determine whether the impact of drought can be influenced by some site conditions such as altitude, slope and substrate. We also attempt to highlight a possible correlation between drought and the observed cedar decline during several decades.

MATERIAL AND METHODS

Description of the study area

The study was conducted in the Ouarsenis Massif (North West of Algeria) (Figure 1), which houses two cedar forests: “Theniet El Had” (1000 ha) and “Ain Antar” (500 ha). These are natural stands of Atlas cedar developing in substantially variable environmental conditions. The current climate trend is characterized by a decrease in the annual precipitation by ca. 25% and the increase in temperature throughout of the year (Figure 1). This would have increased the length of the dry period and resulted in a shift of bioclimate from sub-humid to semi-arid.

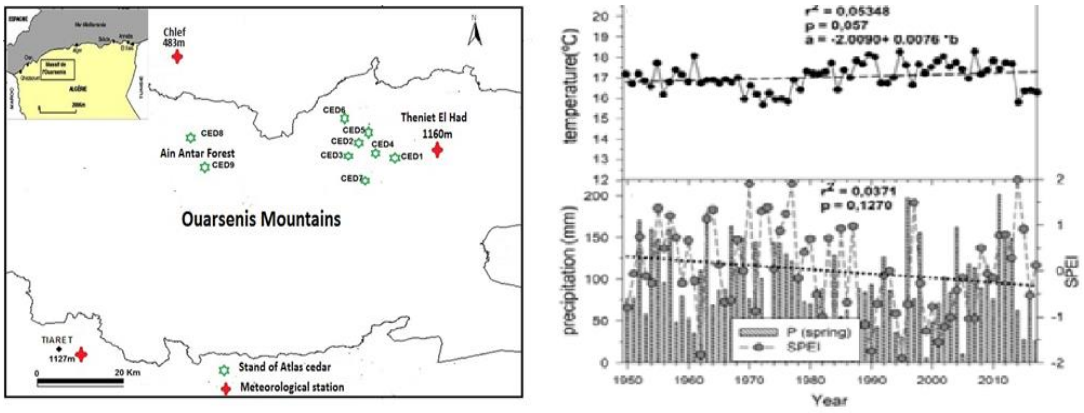

Figure 1. Location of the study area, sites sampled, annual climate trends of mean temperature and total precipitation in the study area and for the period 1950-2010.

Study Material

Nine sites were sampled and analyzed, seven from "Theniet El Had" forest cedar (CED1 to CED7) and two from "Ain Antar" (CED8 and CED9). Site selection was based on the following variables: altitude, slope, exposure and soil type (Table I). At each site, 15 trees from dominant and co-dominant trees were selected and sampled. Two cores were extracted from each tree at a height of 1.30m. The collected samples were processed and analyzed according to standard procedures in dendroecology (Fritts et Swetnam, 1989).

Data processing and analysis

Following fine-sanding, the cores were crossdated by visual comparison under a dissecting microscope using standard dendrochronological techniques (Fritts, 1976).Ring widths were measured using a LINTAB6 measuring table with a resolution of 0.001 mm and the TSAP software (Rinntech ®)to obtain elementary core ring-width series.

Chronology descriptive statistics

For each series, the statistical parameters traditionally implemented in dendrochronology were calculated (Fritts, 1976; Cook et Kairiukstis, 1990):

Expressed population signal (EPS): an EPS value at least 0.85 constitutes an indicator of agreement of the simple chronology variance with that of theoretical population chronology.

Mean sensitivity (MS): calculated for each individual tree and site chronology, considering the absolute relative difference in width from one ring to the next. According to Fritts (1976), threshold of 0.20 defines sensitive (MS >0.20) and complacent (MS<0.20) population.

First-order autocorrelation coefficient (AC): used to estimate the degree of persistence in time series, by calculating the correlation between the time series lagged in time with a lag equal to one year (Fritts, 1976).

Table 1. Site description, geographic coordinates, elevation, aspect, slope, soil and samples. The cedar forest of "Theniet El Had" contains 07 sites (CED1 to CED7) and that of "AinAntar" two sites (CED8 and CED9).

Sites	Coordinates	Elevation (m)	Exposure	Slope %	Soil type	Trees (cores)
CED1	35°53'23''N2°00'02''E	1460	NNE	20-30	Vertisoil	15 (29)
CED2	35°51'44''N1°59'18''E	1520	NE	50-60	Colluvial soil	15 (27)
CED3	35°51'08''N1°57°08''E	1700	N	50-60	Colluvial soil	15 (28)
CED4	35°52'06''N1°58'01''E	1325	NE	10-20	Colluvial soil	15 (30)
CED5	35°52'24''N1°58'25''E	1420	NNW	30-40	Colluvial soil	15 (25)
CED6	35°52'35''N1°56'00''E	1420	NW	20-30	Colluvial soil	15 (30)
CED7	35°51'50''N1°57'27''E	1550	SW	50-60	Colluvial soil	15 (23)
CED8	35°53'34''N1°39'25''E	1150	NW	20-30	Lithosoil	15 (29)
CED9	35°53'30''N1°38'48''E	1450	NNW	50-60	Lithosoil	15 (27)

Rings-width standardization

The intent of standardization is to remove non-climatic age trends from ring-width series (Cook *et al.*, 1990). In this paper, we applied two standardization methods; the first one uses a detrending filter with a 20-year window (low-pass filter) to generate indexed series for each population. The second involves Autoregressive modeling (ARMA) and the bootstrapped method to calculate response functions (Guiot, 1986). Standardizations methods were applied using the package PPPbase (Guiot et Goeury, 1996).

Climate-growth relationship

To understand the relationship between tree growth and climate, response functions were developed (Fritts, 1976; Cook et Kairiukstis, 1990). An autoregressive model (ARMA) was selected to prewhiten the series before computing bootstrapped response functions using the package PPPBase (GuiotetGoeury, 1996).

Climate regressors included monthly precipitation (P),maximal (TM) and minimal temperatures (Tm) over a period of 12 months from October of year *t-1*toSeptember of year *t*. Climate data used are those of meteorological stations of Tiaret (1936-2010).

Results with the highest correlation coefficients and statistical significance merit further interpretation. Statistical significance depends on the "R / S" ratio which expresses the ratio between the correlation coefficient of the period of verification and its standard deviation. Based on the "R / S" value, four significance levels are defined (Table II). Only response functions whose "R / S" is greater than or equal to 1.96 are significant ($p>0.05$) and will be discussed in terms of tree-ring to climate relationships.

Table 2. Overall significance of the response functions. (R/S is the ratio between the correlation coefficient of the period of verification and its standard deviation). Are considered significant, the response functions where R / S> 1.98 (95%).

R/S	Code	signification
1.65-1.96	1	90%
1.96-2.58	2	95%
2.58-3.29	3	99%
>3.29	4	99.9%

RESULTS AND DISCUSSION

Ring-width chronologies

The samples were successfully crossdated. During this step, we found difficulties related to the presence of very narrow, incomplete or missing rings, especially near the bark (Figure3). False rings are very rare in chronologies of Atlas cedar. Raw ring-width series show a clear decreasing trend with age and diameter increase. The mean site chronologies exhibit similar growth patterns emphasizing growth increase sequences (1883-1891, 1909-1912, 1928-1932, 1972-1976) and growth decrease sequences (1867-1872, 1876-1881, 1920-1925, 1942-1947, 1958-1961, 1983-1994, 1999-2002). These variations are common to Atlas cedar populations and caused by a common factor (e.g.climate).

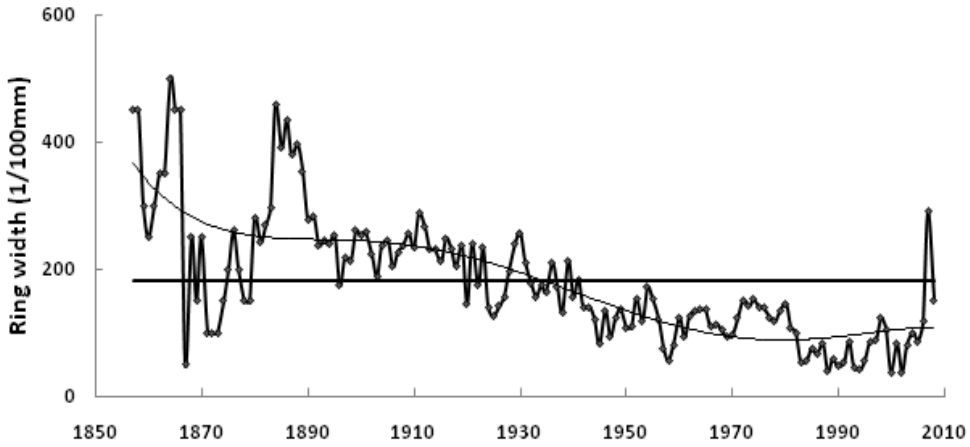

Figure 2.Example of ring-width chronologies of Atlas cedar in Ouarsenis Mountains.

Chronology descriptive statistics

Expressed population signal (EPS) values exceed the threshold of 0.85, suggesting the samples are capturing a common signal at the site. These results support strong correlation and coherence between the chronologies (Table III).

The mean sensitivity (MS) ranges from 0.18 to 0.43 for the individual series and from 0.17 to 0.30 for site chronologies (TableIII). Higher values of mean sensitivity (MS>0.20) are recorded especially at low elevation and/or sandstone and marl substrates (CED1, CED2, CED3, CED4) and high slope (CED9).

First-order autocorrelation coefficients (AC) range from 0.13 to 0.20, indicating a low dependence of current growth on previous year's growth. The longest tree-ring series is 221 years (CED8) and it covers the period 1789-2010.

Radial growth of Atlas cedar in the Ouarsenis Massif seems to be modulated by several environmental factors, including climate, elevation, slope and soil. These factors operate mainly on the water balance in the soil and hence the length of the growing season (Khatouri et Denis; 1990). Considering the agreement between the growth curves of several stands, we can suggest that the factors that govern Atlas cedar radial growth are common and mainly related to climatic factors. These studies support results obtained for this species in Morocco (Till, 1986) and southeastern France (Guibal, 1985).

Table 3. Statistical parameters calculated for each site (chronology; Expressed population signal: EPS; mean sensitivity: MS; first order autocorrelation coefficient).

Sites	Chronology (age)	EPS	Cores MS (Mean)	Site MS	AC
CED1	1857-2010 (153)	0.94	0.25-0.40 (0.32)	0.25	0.162
CED2	1850-2010 (160)	0.87	0.18-0,36 (0.25)	0.19	0.158
CED3	1877-2010 (133)	0.89	0.19-0,27 (0.22)	0.17	0.173
CED4	1930-2010 (81)	0.93	0.29-0.43 (0.33)	0.30	0.203
CED5	1870-2010 (140)	0.89	0.25-0.38 (0.29)	0.27	0.171
CED6	1860-2010 (150)	0.95	0.25-0.41 (0.32)	0.29	0.162
CED7	1891-2010 (119)	0.89	0.19-0.28 (0.23)	0.19	0.186
CED8	1789-2010 (221)	0.92	0.21-0.29 (0.24)	0.20	0.167
CED9	1866-2010 (144)	0.87	0.22-0.29 (0.25)	0.21	0.134

Climate-growth relationship

In all populations precipitation from October$_{t-1}$ to April$_t$ positivelycorrelates ring-width. Growth is also positively relatedto August and September precipitation. In contrast, negative relationships are found between May to June precipitation and ring width. Lowland populations (CED1, CED4, CED5 and CED6) are more sensitive to precipitation throughout the year. Minimal temperatures are positively

related to radial growth in December and January (CED1, CED3, CED7, and CED9), February (CED2, CED4, CED5, CED6 and CED7) and March (CED5 and CED6) but negatively in November (CED5 and CED7). As for maximum temperatures, they are negatively related to ring width, especially in summer (Figure3).

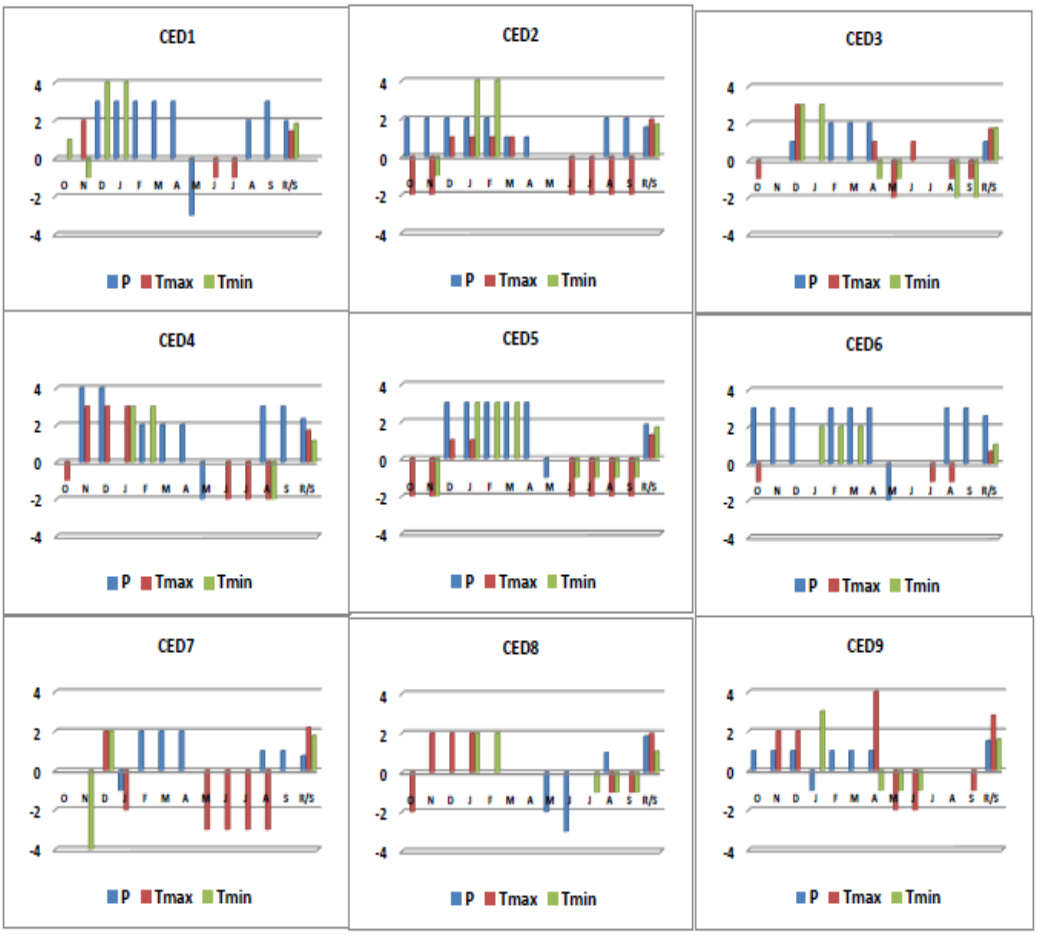

Figure 3. Response functions of Atlas cedar at the 9 sites (1936-2010). Bootstrap values (v) are significant at v> 2. The black bar indicates the relationship with monthly precipitation (P), stoneware with maximum temperatures (Tmax) and the white with the minimum temperatures (Tmin)

Atlas cedar shows a positive relation with precipitation, especially those that precede and coincide with the ring formation. This relationship shows that cedar radial growth is mainly limited by water supply during both the months prior to cambial activity (November to February) and the period of cambial activity (March-April).The positive relation with winter precipitation could be attributed to

water storage in the soil for use during the growing period (Till, 1986).An ongoing cambial activity from August to September could be a possible cause of the positive relationship shown between precipitation and ring width (Ladjal *et al*., 2007).Temperature, perhaps play a decisive role in Atlas cedar radial growth. Its role is positive in winter but negative in summer. The positive role of winter temperatures can be explained by its influence on photosynthesis during mild sunny winter days. In contrast, the negative effect of high temperatures during the summer underlines the importance of water balance deficit as a result of low precipitation and high temperature (Till, 1986). Touchan *et al*. (2017) showed that Atlas cedar has positive relationships with winter and spring precipitation and a negative response with spring and summer temperatures. In addition, compared to other species in the study area (Sarmoum *et al*., 2016; Hibbani et Abdoun, 2018), Atlas cedar growth is closely related to precipitations and appears more sensitive to drought. We found also that populations located at low altitude and/or marl and sandstone substrate are more sensitive to precipitation. This sensitivity is demonstrated by high mean sensitivity coefficients (MS) and a positive relation with precipitation throughout the year.

Impact of drought on radial growth and stands health

The dry years cause a sudden and prolonged decrease in soil water reserves and expose trees at a high risk of water stress, which finds its peak in summer (Ladjal *et al*., 2007). Several studies have shown that site characteristics help to increase the effect of drought (Kane *et al.*, 2014). We suggest that responses of growth to drought were conditioned by the local site characteristics related to the variability in soil water-holding capacity.The effects of water stress on trees are multiple: combination of a high water deficit and high evapotranspiration may cause a malfunction of the water supply and lead to xylem embolism and cavitation (Meinzer et McCulloh 2013).In species well adapted to high levels of water stress such as Aleppo pine, evapotranspiration is controlled by the opening and closing of stomata when water stress occurs (Borghetti *et al.*, 1998); in this case, the risk of cavitation and embolism of xylem is limited (Martinez-Vilalta et Pinol 2002). However, Atlas cedar continues its physiological activities under high levels of water stress, which increase the risk of xylem embolism (Ladjal *et al.*,2007; Gaba-Chahboub *et al.*, 2016).The vulnerability of xylem may also change with the age of the tree; older trees are more vulnerable to xylem embolism which could contribute to a gradual loss of functionality of the xylem and thus the mortality of the tree (Linares *et al.*, 2013).Loss of the tree vitality due to water stress decreases tree resistance to pathogens and parasite attacks (Raouault *et al.*, 2006). It therefore seems logical that after long periods of drought, high mortality in forest stands is observed (Cailleret *et al.*, 2014). In the future, the importance of drought as a limiting growth factor is expected to increase in North Africa (Barkhordarian *et al.*, 2013) and extreme events such as droughts will be more frequent (Vizy et Cook 2012). The vitality of Atlas cedar seems to be conditioned by the frequency of drought years.

CONCLUSION

This work was able to highlight the relationship between drought and site characteristics to explain the decline in growth and the loss of vitality in Atlas cedar in the Ouarsenis massif. As a result, the years of drought adversely affected cedar growth, particularly on marl and sandstone substrate and/or low altitude, where massive tree mortalities were recorded over the years 1983, 1984, 1988, 1994 and 2002. It is necessary to establish a surveillance network in all cedar forest affected by decline.

ACKNOWLEDGMENTS

This work was funded by the Algerian Minister of Higher Education and Scientific Research (PNR project). We are also very grateful to Abdelkader MASLOUB from the National Park of Theniet El Had, Algeria, for his valuable assistance during the fieldwork and Khaled TAÏBI from the Ibn Khaldoun University of Tiaret, Algeria, for the revision of the manuscript.

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Original scientific paper
10.7251/AGRENG1903053G
UDC 634.73

GENETIC DIVERSITY STUDIES OF LATVIAN *VACCINIUM MYRTILLUS* L. POPULATIONS FOR *IN SITU* CONSERVATION

Agnese GAILĪTE*, Anita GAILE, Dainis RUŅĢIS

Genetic Resource Centre, Latvian State Forest Research Institute "Silava", Latvia
*Corresponding author: agnese.gailite@silava.lv

ABSTRACT

Plants and berries of bilberries (*Vaccinium myrtillus* L.) are traditionally used in many nations as a local medicine as well as edible plants. They are an important feed source for wild animals and birds. *In situ* conservation is an important component for the conservation of crop wild relatives (CWR) and wild harvested plants (WHP). Research on population structure and genetic diversity is important and is required for the development and implementation of *in situ* conservation strategies as well as being useful for ecosystem services management. The aim of this study was to test EST-SSR markers for bilberry genotyping and determine genetic diversity in different forest types – *Vacciniosa, Myrtillosa, Hylocomiosa* as well as compare populations from various regions of Latvia. Our results indicated that there was a small genetic differentiation between bilberries grown in different forest types (0-2%); most of the variation was found within individuals. Analysing populations in different regions of Latvia, 5% of the genetic variation was found among populations. Analysis using the STRUCTURE software package showed that there were no isolated populations or distinct groups. There was a positive correlation between geographic and genetic distances, indicating that the analysed populations differentiation can be explained by isolation-by-distance, without additional dispersal barriers.

Key words: *Vaccinium myrtillus, genetic diversity, in situ conservation.*

INTRODUCTION

Vaccinium myrtillus L. (bilberries) are wild plants traditionally used as a local medicine as well as edible plants. It is a woody dwarf shrub (5-90 cm high) typical in the northern hemisphere (Nestby et al., 2010) and can form clonal colonies or patches up to 15 metres in diameter (Ritchie, 1956).

In situ conservation has become increasingly recognised as an important component of conservation strategies. Plant species conserved *in situ* are an essential source for breeding and development of new varieties (Zoratti et al., 2015). Systematic research and conservation activities for crop wild relatives (CWR) and wild harvested plants (WHP) have been initiated in many European countries. Population and genetic diversity studies are important sources of

information for the development and implementation of conservation strategies. The main CWR and WHP plant groups in Latvia are forage grasses, aromatic and medicinal plants, and forest fruits and berries, therefore molecular studies of these plants could play an important role in the development of conservation strategies as well as providing information for ecosystem services management.

Latvia is located in the temperate climatic zone with a range of habitats with differing ecological parameters. The amount of rainfall and the depth of snow cover on the highlands is higher, especially on the western slopes. Over the whole territory of Latvia, in the direction of west to east there is a decreasing influence of the Atlantic Ocean and the Baltic Sea, and an increase of climatic continentality, which determines the grouping of Latvian nature districts into regions (Kavacs, 1995). According to FAO data (2015) 54% of Latvia is covered by forests and bilberries are widespread within forests. There are dry site type forests, forests on wet mineral soils, forests on wet peaty soils, forests on drained mineral soils and forests or former bogs on drained peaty soils in Latvia (Zālītis, Jansons, 2013). *V. myrtillus* reaches its maximum development in pine-dominated sites (Ritchie, 1956), therefore all samples for DNA analysis were collected from pine forests.

The majority of molecular studies, including the use of EST-SSR markers, have been done on species of the section *Cyanococcus* (Boches et al., 2005; Rowland et al., 2003). The species endemic to Latvia belong to other section (Nestby et al., 2010), therefore the available DNA markers need to be tested and adapted for use in *V. myrtillus*. In addition, there are only a few studies on genetic diversity of bilberries, including studies with using inter-simple sequence (ISSR) markers (Zoratti et al., 2015). Clonal structure of bilberry was studied with RAPD and AFLP markers (Albert et al., 2003; Albert et al., 2004), mating system and genetic structure – with isozymes (Jacquemart et al., 1994).

The aim of this study was to test EST-SSR markers for bilberry genotyping and determine genetic diversity in different forest types, in different locations.

MATERIALS AND METHODS

During the growing season, bilberry leaves were collected in three types of dry site type forests (*Vacciniosa*, *Myrtillosa*, *Hylocomiosa*) in seven locations of Latvia (Fig. 1).

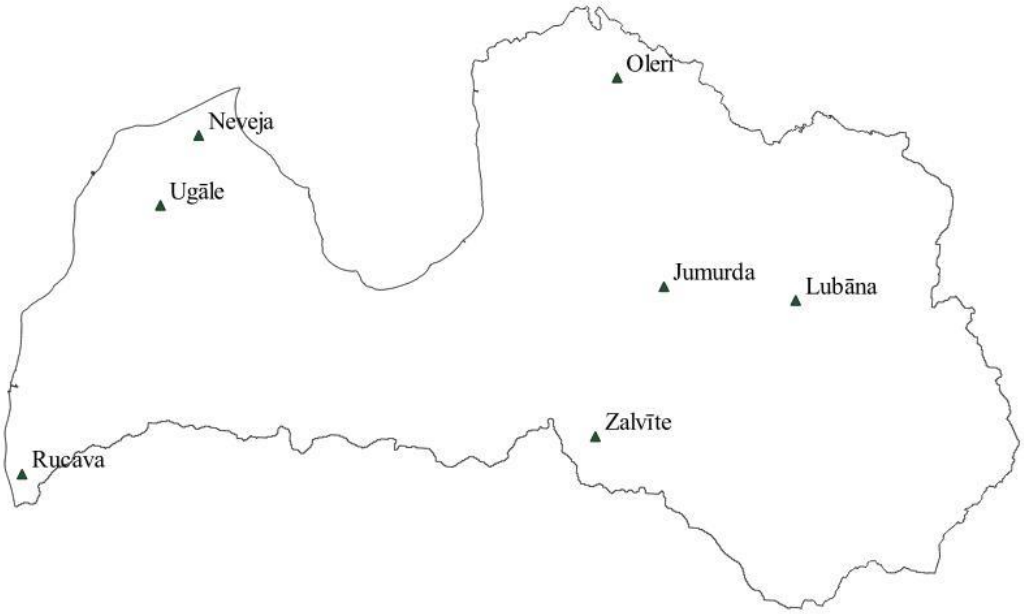

Fig.1. Locations of collected samples.

In each location, three forest types (sites) were analysed. In each site, at least 24 stems with leaves were collected. The distance between collected samples was approximately 15 metres. DNA was extracted using a modified CTAB method (Doyle, Doyle, 1990). From 18 tested EST-SSR markers (Boches et al., 2005), analyses were performed with eight markers – Na741, CA236, CA421, CA112, CA483, NA 961, VCC_K4, VCC_J5 (Table 1) labelled with one of three fluorophores (6-FAM, HEX or TAMRA). PCR reactions were performed in a volume of 10 µl containing approximately 50ng DNA, 1µl HOT FIREPol® 10x Buffer B2 (Solis BioDyne), 2 mM $MgCl_2$, 0.2 mM dNTP mix, 0.4 µM forward and reverse primers. PCR was carried out in a thermocycler (Eppendorf Mastercycler epgradient): initial denaturation at 95 °C for 20 min, followed by 30 cycles at 94°C for 30 sec, annealing temperature of the primer pair (Table 1) for 45 sec, 1 min 72°C and a final extension at 72°C for 10 min.

Table 1. Markers utilised for analyses.

Locus	Annealing temperature, ºC	Allele size range (bp)	Number of alleles
Na741	58	310-320	5
CA236F	60	231-247	2
CA421F	60	183-247	23
CA112F	58	170-190	4
CA483F	58	312-340	8
NA 961	60	174-198	6
VCC_K4	60	201-265	13
VCC_J5	54	266-330	29

The PCR fragments were visualised on an Applied Biosystems ABI Prism 3100xl Genetic Analyser. Genotyping was performed using GeneMapper 4.0. (Applied Biosystems). Genotype data were analysed with programs GenAlEx 6.501. (Peakall, Smouse, 2012), Micro-Checker 2.2 (Van Oosterhout et al., 2004), STRUCTURE 2.3.4. (Pritchard et al., 2000).

RESULTS AND DISCUSSION

Of the seven analysed markers, two (VCC J5 and NA 961) had high fixation indices (0.66 and 0.76 respectively), indicating inbreeding or the presence of null alleles. The presence of null alleles can be expected due to the use of cross-species SSR markers. The EST-SSR markers (NA 741, NA 961, CA 421F and CA 483F) were reported to be successfully used for bilberry genotyping (Dahlǿ, 2011), in contrast to our results with marker NA961 showing increased $F_{is,}$ Therefore the use of these two markers (VCC_J5 and NA 961) for analysis of Latvian bilberry populations should be further assessed.

The number of alleles detected by the utilised markers ranged from 2 (CA 236F) to 29 (VCC_J5) (Table 1). The effective number of alleles varied from 2.384 to 3.381. The mean number of private alleles, unique to one population is rather small - 0.25 to 0.75. No locally common alleles present in less than 25% of populations were found, and locally common alleles present in less than 50% of populations was also low within each population (1.375-1.625). The mean Information index (I) in each population, which is equivalent to the Shannon-Weaver index, varied between 0.984 to 1.208 (Table 2).

Table 2. Mean allelic patterns across populations.

Population	Zalvīte	Neveja	Rucava	Oleri	Lubāna	Ugāle	Jumurda
Na	7.750	7.500	6.875	6.750	6.250	7.875	6.750
Na Freq. >= 5%	3.375	3.000	2.875	3.375	3.250	3.375	3.250
Ne	3.381	2.678	2.786	2.730	2.384	3.055	2.903
I	1.208	1.044	1.058	1.077	0.984	1.155	1.119
No. Private Alleles	0.250	0.750	0.125	0.250	0.000	0.375	0.250
No. locally common alleles (<=25% of pops)	0.000	0.000	0.000	0.000	0.000	0.000	0.000
No. locally common alleles (<=50% of pops)	1.375	1.250	1.500	1.375	1.625	1.500	1.375
He	0.558	0.479	0.501	0.513	0.470	0.524	0.526

Na – number of alleles; Ne – effective number of alleles; I – Information index; He - expected heterozygosity.

Each location was analysed separately. Analysis of molecular variation (AMOVA) indicated only a small difference (0-2%) between populations growing in different

forest types. Analysis of each forest type in different locations, indicated that most of the variation was found within individuals (63-76%) and among individuals (24-36%). As *V. myrtillus* is a long-lived species with a mixed breeding system (Jacquemart et al., 1994) it is clear that most of genetic variation was found within individuals. Similar results were obtained previously, using RAPD markers to investigate population in differing habitats (Alberts et al., 2004), where genotypic diversity indicators did not vary significantly between habitats.

AMOVA indicated that 5% of molecular variance was found between populations in different locations in Latvia, 31% of the variation was found among individuals and 64% within individuals. STRUCTURE analysis did not identify any population substructure or isolated populations.

Comparison of pairwise genetic and geographic distance matrices show that there is a positive and significant ($p<0.001$) correlation between geographic and genetic distances (Fig. 2).

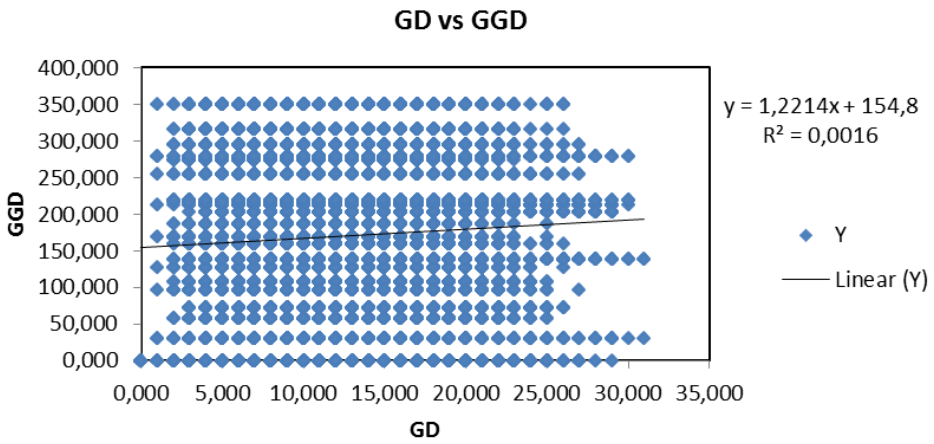

Fig. 2. Correlation between genetic (GD) and geographic (GGD) distances.

A similar significant correlation between geographic and genetic distances, indicating isolation by distance, was previously reported in Norwegian, Finnish, Icelandic and German genotypes (Zoratti et al., 2015).

Genetic diversity is essential for evolution and adaptation over long time (Sgrò et al., 2010). These results indicated that the Latvian bilberry populations were not highly differentiated, and the small number of unique alleles in each population were rare, low frequency alleles.

CONCLUSIONS

Our results indicate that genetic differentiation of Latvian bilberry populations is not influenced by forest type. The utilised markers did not identify genetically unique populations. The genetic differentiation of bilberry populations growing in different regions of Latvia is most likely a result of isolation by distance.

Therefore, the correlation between genetic and geographic distances should be taken into account when developing an *in situ* conservation strategy for bilberries and other forest berry species in Latvia.

ACKNOWLEDGEMENTS

This work was supported by the European Regional Development Fund Postdoctoral research aid Nr.1.1.1.2/VIAA/1/16/123 "Investigation of *Vaccinium* genetic resources in Latvia". Many thanks to our colleagues from Genetic Resource Centre: Anna Korica, Baiba Krivmane, Krišs Bitenieks and Viktorija Beļeviča for assistance in DNA extraction.

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Original Scientific paper
10.7251/AGRENG1903060U
UDC 633.15

INFLUENCE OF INTERCROPPING MAIZE WITH CLIMBING BEAN ON FORAGE YIELD AND QUALITY

Darko UHER[1], Zlatko SVEČNJAK[1], Dubravka DUJMOVIĆ-PURGAR[1], Dario JAREŠ[1], Ivan HORVATIĆ[2*]

[1]Faculty of Agriculture, University of Zagreb, Zagreb, Croatia
[2]Božjakovina d.d., Božjakovina, Croatia
*Corresponding author: ihorvatic@hotmail.com

ABSTRACT

Maize forage is poor in protein content which shows its low quality and nutritive value. Regarding to high feed costs of protein supplementations, legumes can be used in livestock nutrition for their high protein content and, thus, providing cost savings. Since legumes have low dry matter yield, acceptable forage yield and quality can obtained from intercropping cereals and legumes compared with their sole crops. In this study, maize (*Zea mays* L.) and climbing bean (*Phaseolus vulgaris* L.) were intercropped in different sowing densities and their monocropping equivalents were tested to determine the best intercropping system on forage yield and quality. Maize was cultivated alone (75 000 plants ha^{-1}) and intercropped with bean as follows: 75 000 plants ha^{-1} of maize and 37 500 plants ha^{-1} of bean (MB_1), 75 000 plants ha^{-1} of maize and 50 000 plants ha^{-1} of bean (MB_2) and 75 000 plants ha^{-1} of maize and 75 000 plants ha^{-1} of bean (MB_3), in rows alternating with maize. The highest dry matter yield was produced by MB_3 (20.9 t ha^{-1}), and the lowest by maize (16.9 t ha^{-1}). All intercropped systems had higher crude protein contents, MB_1 (92 g kg^{-1} DM), MB_2 (99 g kg^{-1} DM) and MB_3 (110 g kg^{-1} DM), than the maize (77 g kg^{-1} DM). Intercropping of maize with bean reduced neutral and acid detergent fiber, resulting in increased forage digestibility. Therefore, maize intercropping with bean could substantially increase forage quantity and quality, and decrease requirements for protein supplements as compared with maize.

Keywords: *Intercropping, Maize, Climbing Bean, Yield, Quality.*

INTRODUCTION

In many regions of Europe, whole-plant maize silage is the basic feed used in feeding cows and fattening cattle. Despite its high energy content, the protein content is low (88 g kg^{-1}) compared with legumes silage (Anil et al., 2000) and needs to be supplemented with proteins for better feed quality (Stoltz et al., 2013). Intercropping maize with legumes for silage is a feasible strategy to improving the level of crude protein (Contreras-Govea et al., 2009; Zhu et al., 2011). Although

maize provides high yield in terms of dry matter, it produces low protein content in fodder. The bean (*Phaseolus vulgaris* L.) is a common legume cultivated for its edible seeds all over the world. It is slightly hairy with a well-developed root system and the stems are many branched. The bean is a fast growing, warm season legume, and, it can grow in a diverse range of environmental conditions worldwide because of its adaptability. There are many varieties of beans grown in all the regions. However, selecting high yielding (seed and herbage), disease resistant variety is most important factor for successful cultivation. In addition, the bean serves as an adequate source of protein. Furthermore, it can be planted alone or intercropped with other crops such as corn and sorghums. Javanmard et al (2009), worked on intercropping of maize with different legumes, and showed that dry matter yield and crude protein yield of forage were increased by all intercropping compositions compared with the maize monocrop. Physiological and morphological differences between intercrop constituents influence their ability to use resources; especially cereals with legumes, have several advantages such as higher overall yields, better soil utilization (Dhima et al., 2007), yield stability of the cropping system (Lithourgidis et al., 2006), better use of light, water and nutrients (Javanmard et al., 2009), improved soil conservation (Anil et al., 1998), soil fertility through biological nitrogen fixation, increases soil conservation through greater soil coverage as compared to sole cropping, and ensures better soil-susceptible crop in monoculture (Lithourgidis et al., 2006), and better control of pests and weeds (Banik et al., 2006; Vasilakoglou et al., 2008). Atmospheric nitrogen fixation using legumes plants can reduce nitrogen competition in the reciprocal intercropping system of legumes and cereals enabling the cereals to use more nitrogen in the soil (Eskandari et al., 2009). This can affect the quality of the fodder intercrop components because the protein content is directly related to the content of nitrogen in the forage plants (Putnam et al., 1985).

This study was designed to determine the influence of different patterns of maize-climbing bean intercropping on the yield and quality of forage.

MATERIAL AND METHODS

A field experiment was carried out during the 2017 growing season at experimental fields in Daruvar (45°35'34″N, 17°13'25″E), Croatia. Meteorological data of the experimental site are presented in (Table 1).

Table 1. Air temperature and rainfall by month during the 2017 growing season.

Meteorological data	Month					
	April	May	June	July	August	September
Air temperature (°C)	10.9	16.5	21.8	22.9	22.4	14.7
Rainfall (mm)	62.8	45.0	70.3	71.9	29.0	121.7

The experiment was set up as a randomized complete block design with three replicates. Maize hybrid seed (KWS Kolumbaris) was obtained from Seed Company "KWS". Seed of the climbing bean cultivar "Meraviglia Di Venezia" was obtained from Company "Green Garden". The treatment comprising the individual plot size was 50 m × 2,8 m. The maize population 75 000 plants ha^{-1} (SM) were spaced at 70 cm × 19 cm and climbing bean population 37 500 (MB_1), 50 000 (MB_2) and 75 000 plants ha^{-1} (MB_3) were spaced at 70 cm × 38.1 cm, 70 cm x 28.6 cm and 70 x 19 cm, respectively, in rows alternating with maize. Basic tillage was carried out by ploughing to 30 cm depth. Presowing preparation was done using a tractor-mounted rototiller. All plots were fertilized with the same amount of fertilizer before sowing, containing 200 kg of N ha^{-1}, 100 kg P_2O_5 ha^{-1} and 200 kg of K_2O ha^{-1}. Maize and climbing bean were sown to a depth of approximately 5 cm by maize drill in May 3, 2017. Herbicide Wing P (active substance 212.5 g/l dimethenamide-p and 250 g/l pendimethalin) was applied pre emergence in intercropping maize with climbing bean at a dose of 4 l ha^{-1}. The soil of the research area has an acid pH 4.4 reaction (M-KCl), good humus (3.3%), poorly supplied with physiologically active phosphorous (7.6 mg P_2O_5/100 g soil), medium supplied with physiologically active potassium (22.3 mg K_2O/100 g soil) and richly supplied with total nitrogen amounting to 0.15%. The crops were harvested when the maize reached at soft dough stage and climbing bean at R7 stage and then chopped into 20 mm size pieces with a chaff cutter. The dry matter content was determined by drying in an oven at a temperature of 65°C to a constant mass. Crude protein was measured according to Kjeldahl (AOAC, 2000), neutral and acid detergent fibres according to Van Soest et al. (1991), calcium, potassium were analysed by atomic absorption spectrophotometry by analyzer Spectrophotometer 2010 Model M530 Infrared Spectrophotometer (USA) and phosphorus was analysed by colorimetry (AOAC, 2000). The water soluble carbohydrate (WSC) was determined by the anthrone method, using freeze dried samples, where the WSC was extracted with water (Tomas et al., 1977). Statistical analyses: Analyses of variance were made for fresh forage and dry matter yield and forage quality parameters ($P<0.05$), and the Tukey test was used for comparing means ($P<0.05$). Data were analyzed using SAS statistical software (SAS Inst., 2002).

RESULTS AND DISCUSSION

Table 2 shows the yield of forage and dry matter of maize intercropped wih climbing bean. The diferences in the yield of forage and dry matter are statistically significantly ($P<0.05$). The yield of forage and dry matter yield ranged from 66.3 t ha^{-1} (MB_3) to 46.7 t ha^{-1} (SM) and 20.9 t ha^{-1} (MB_3) to 16.9 t ha^{-1} (SM). According to the results, when climbing bean seed number increased in intercrop, forage and dry matter yields on parcels increased. The intercropped maize with cowpea (*Vigna unguiculata* (L.) Walp.) and bean (*Phaseolus vulgaris* L.) produced higher dry matter yield than monocrop maize (Geren et al., 2008). Dry matter concentration was in the range recommended for ensiling of maize-climbing bean intercropped.

One of the main reasons of intercropping maize and climbing bean is the increase crude protein level in silage.

Table 2. Fresh forage and dry matter yield of maize and maize-climbing bean intercropped

Intems	Treatmens			
	SM	MB_1	MB_2	MB_3
Fresh forage yield in t ha^{-1}	46.7^{c}	53.1^{bc}	59.5^{ab}	66.3^{a}
Content of dry matter in g kg^{-1}	361^{a}	343^{b}	326^{c}	315^{d}
Dry matter yield in t ha^{-1}	16.9^{c}	18.2^{b}	19.4^{b}	20.9^{a}
Crude protein yield in t ha^{-1}	1.30^{d}	1.67^{c}	1.92^{b}	2.30^{a}

Means within a row marked with different letters are significantly different at ($P<0.05$).

Since crude proteins are very important in cattle fodder, silage containing more crude proteins is desirable. In this study it was found that the value of crude proteins of intercropped fodder MB_1, MB_2 and MB_3 was statistically significantly ($P<0.05$) higher than SM (Table 3). According to the results, when climbing bean seeds number increased in intercrops, the content of crude protein in the mixture increased. Armstrong et al. (2008) found that climbing bean intercropped with corn had the greatest potential among the climbing beans to increase crude protein concentration compared with monoculture corn. The intercropping of maize (*Zea mays* L.) with climbing bean (*Phaseolus vulgaris* L.) may serve as a way to increase crude protein and improve the overall nutritive value of silage (Grobelnik et al., 2005). Results in the present study were in agreement with other studies where legumes also increased crude protein concentration when in a mixture with maize (Htet et al., 2016; Dawo et al., 2007). In this study it was found that the yield of crude proteins of intercropped fodder MB_1, MB_2 and MB_3 was statistically significantly ($P<0.05$) higher than SM (Table 2).

The results suggested that the contributions provided by legume components in the mixtures increased crude protein yields of fodder. This could be due to higher nitrogen availability for maize in intercropping compared with the monoculture crop (Eskandari et al., 2009). From this point of view fodder produced in maize-climbing bean intercrops is important not only to profit from the increase in the content of crude protein, but also from the reduction of the content of neutral and acid detergent fibers.

Table 3. Nutrient composition of maize and maize-climbing bean intercropped fresh forage (g kg^{-1} dry matter)

Nutrient composition	Treatmens			
	SM	MB_1	MB_2	MB_3
Crude protein	77^{d}	92^{c}	99^{b}	110^{a}
Neutral detergent fiber	370^{a}	353^{b}	348^{c}	334^{d}
Acid detergent fiber	203^{a}	189^{b}	179^{c}	170^{d}
Potassium	5.6^{b}	6.3^{a}	6.5^{a}	6.9^{a}
Phosphorus	2.4^{c}	2.5^{bc}	2.6^{ab}	2.7^{a}
Calcium	3.4^{d}	3.8^{c}	4.1^{b}	4.5^{a}
Water soluble carbohydrate	136^{a}	118^{b}	113^{bc}	103^{c}

Means within a row marked with different letters are significantly different at ($P<0.05$).

For this reason, the best option in maize-climbing bean intercropping is the use of climbing bean genotypes that provide forage with the greatest amount of pods at harvest. In addition, the level of neutral detergent fibers is associated with the stage of maturity of the fodder due to the level of the cell wall components, mainly cellulose, hemicellulose and lignin (Mugweni et al., 2000). The value of a neutral detergent fiber refers to the total cell wall and consists of an acid detecting fiber fraction plus hemicellulose. In this study it was found that the values of neutral and acid detergent fibers of intercropped MB_1, MB_2 and MB_3 were statistically significantly ($P<0.05$) lower than SM (Table 3). According to the results, when climbing bean seed number increased in intercrop, the values of neutral and acid detergent fibers in the mixture decrase. The content of neutral detergent fiber is important in ration formulation because it reflects the amount of animal forage that animals can consume (Lithourgidis et al., 2006). In general, the concentration of neutral detergent fibers is higher for grass than for legumes (Dahmardeh et al., 2009). Many researchers stated that the nutritional value of cell wall components decreased with plant age was related to increased lignin content (Atis et al. 2012; Zhao et al., 2012). Since smaller amounts of fiber components are used for better digestion, the climbing bean intercropped plots to be superior to monocrop maize in terms of neutral detergent fiber. In this paper, the value of water soluble carbohydrate (WSC) of intercropped forage MB_1, MB_2 and MB_3 was statistically significantly ($P<0.05$) lower than SM (Table 3). According to the results, when the climbing bean seed number increased in intercrop, the values of water-soluble sugar in the mixture decrase. Contreras-Govea et al. (2011) ensiled corn and forage sorghum with different proportions of lablab bean and reported that legume must make up at least 50% of the mixture to affect fermentation and nutritive value. In this paper, the value of potassium, phosphorus and calcium of intercropped forage MB_1, MB_2 and MB_3 was statistically significantly ($P<0.05$) higher than SM (Table 3). According to the results, when the climbing bean seed number increased in intercrop, the values of potassium, phosphorus and calcium in the mixture

indecrase. Contribution of legumes with sweet sorghum in mixtures was significant increased potassium, phosphorus, calcium and magnesium in fresh fodder (Terzić et al., 2004; Basaran et al., 2017).

CONCLUSION

The conclusion of the present study is that intercropping of maize with climbing bean at various planting densities was shown to be an effective way to influence fresh biomass production, dry matter and crude protein yield to enhance nutrient quality of forage. Intercropping of maize with climbing bean increased values of crude protein, potassium, phosphorus and calcium and decreased values of neutral and acid detergent fibre and water-soluble carbohydrate concentrations in forage. Finally, intercropping with 75 000 plants ha^{-1} of maize and 75 000 plants ha^{-1} of climbing bean was most suitable according to the nutrient composition in forage.

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Original Scientific paper
10.7251/AGRENG1903068M
UDC 316.334.55:314.7

MIGRATION DECISIONS AMONG RURAL HOUSEHOLDS IN RWANDA: WHAT DOES THE PUSH-AND-PULL MODEL REVEAL?

Edouard MUSABANGANJI[1]*, Charles RURANGA[12], Aristide MANIRIHO[1]

[1]School of Economics, College of Business and Economics, University of Rwanda, Rwanda
[2]African Center of Excellence in Data Science, University of Rwanda (ACE-DS/UR), Rwanda
*Corresponding author: musabanganji@gmail.com

ABSTRACT

A country economic status is strongly linked to the transition of its population from one area to another. This, because labor and other forms of migration, has a two-fold advantage: (I) resourcing the targeted location by skilled labor force, and (II) improving migrant households' livelihoods by lowering the vulnerability level. This research aimed at understanding the factors affecting migration decisions among rural households in Rwanda. Data on internal migration were collected in 5033 rural households in 2016/2017 as a part of the fifth nation-wide cross-sectional survey on the Households Living Conditions, and analyzed using the binary logistic regression model. The major findings showed that internal migration was higher in Southern (31.9%) and Western (24.3%) provinces, where official reports pointed out a high level of poverty. The lower rate was observed in Kigali City (3.5%) which was actually considered as richest area and the most internal migration 'pull factors' (jobs and other livelihoods opportunities) offering zone. Results also revealed that, on one hand, being from a rural area, the age, having a large household size, having advanced education level, and being an female household head were the '*push factors*' increasing by around 30% and more the probability of deciding to migrate to another region. On the other hand, owning a land and being reach decreased the likelihood of moving to other zones. This leads to affirm that employment opportunity and availability of diversified livelihoods sources in receiving regions constitute the main '*pull factors*' of migration decisions at rural household level. In light of these findings, it is recommended to (I) ensure more balanced regional growth and opportunities for increased access to off-farm employment for a larger proportion of the rural population and (II) carry out a study on the effects of migration on the livelihoods of migrant-sending households in order to make a thorough and refined situational analysis.

Keywords: *Internal migration, Pull factors, Push factors, Rwanda.*

INTRODUCTION

Rwanda is a landlocked country with an area of 26,338 km^2 and an estimated average population density[1] of 467 and 2,535 inhabitants per km² in rural and urban areas respectively in 2017 (NISR, 2018a). It is ranked among the most densely populated countries in the world. The poverty level has slightly decreased from 39.1 percent to 38.2 percent of the population (NISR, 2018b). In Rwanda, disparities in living standards between lagging and leading areas, or between rural and urban areas, are substantial, and largely correspond with disparities in economic density (World Bank, 2017). The poverty trend report by NISR (2016) reveals that poverty is far lower in urban than in rural areas and almost three times as high as urban poverty and its rate in Kigali is half as high as elsewhere in Rwanda. In 2014, poverty rates in the poorest district were four times higher than poverty in the most economically dynamic district, and poverty in rural areas was twice the rate in urban areas (World Bank, 2017). Services and industries employ only roughly 20 percent of the population, and the primary sector remains the engine of economic growth in Rwanda providing more than 70% of exports in value (Musabanganji, 2017) and accounting for 31% of the Gross Domestic Product (GDP) (NISR, 2018a). For many years, Rwanda has been aware of the role of structural transformation to achieve its objective of promoting macroeconomic stability and wealth creation to reduce aid dependency (MINECOFIN, 2013). This is certified by a series of structural changes implemented since 2000s to fuel the country's ambitions for sustainable economic development advocated by the long-term socio-economic development program known as 'Vision 2020' and subsequent poverty reduction strategies and programs. This has been followed by a series of phenomena within the Rwandan society, including rural-urban migration (World Bank, 2017), and a decrease in poverty and extreme poverty levels especially in rural areas across the country throughout the years (NISR, 2018b). In Rwanda, migration is encouraged by strategic programs that have been put in place in line with the national and regional commitments regarding local and regional free movement of people. It is perceived as a triple win effect due to its far-reaching potential to enhance migrants' wellbeing, while contributing to the development of their communities of origin and destination (IOM, 2015). Rwandan government believes that skilled migrants greatly contribute to national economic development through knowledge transfer especially in skilled labor-demanding sectors made easy by easing local and international movement of highly skilled workforce (MINECOFIN, 2013). Ultimately, labor and other forms of migration play an important role in improving migrants' households' livelihoods by increasing assets, addressing livelihoods challenges and then lowering the vulnerability level. According to Patnaik, Satpathy and Mandal (2014), migration, permanent in nature or temporary, is a physical shifting of an employee or work force from one place to other and it is characterized by various internal dynamic. The decision of household's members to leave their familiar surroundings is due to communities'

[1] These are the 2017 medium scenario projections by the National Institute of Statistics of Rwanda based on 4th Population and Housing Census Projections.

inability to provide physical protection from attack or abuse or to guarantee good public-service delivery and governance at the local and national level, a certain business investment environment, or high employment (Mansoor and Quillin, 2007). In addition, it is asserted by NISR (2016) that the migration of workers from rural to urban areas, from agriculture and other labor-intensive primary activities to industry and services is a phenomenon that is more likely to happen in the course of economic development. In the case of Rwanda, NISR (2014) reveals that about 7 percent of rural dwellers and 23 percent of urban dwellers were recent internal migrants. This is resulting from observed economic growth, rising education levels, and improvements in physical transport infrastructure, combined with increased population pressure on arable land (World Bank, 2017). Scholars (see for instance, Ibourk (2016)) have documented the fact that migration is becoming an increasingly difficult phenomenon to identify and understand. This is the reason behind the observed increase of the studies analyzing the migration motivations and intentions all over the world (Ibourk, 2016, Yorimitsu, 1985). However, in Rwanda, such studies are scarce, especially those intended to understand the factors underlying migration decisions in farm households. Migration is a recent phenomenon in Rwanda (World Bank, 2017), and therefore, deserves particular attention. Thus, this study will contribute in fulfilling this gap by bringing in a thorough understanding of the factors affecting migration decisions among rural households in Rwanda. This study intends to measure the extent to which push and pull factors are affecting the migration decisions in rural Rwanda.

MATERIALS AND METHODS

The study used the fifth integrated household living conditions survey (EICV 5) cross-sectional data collected from October 2016 to October 2017 by the National Institute of Statistics of Rwanda (NISR). The study will use a sample size of 5,035 rural households. Data collection used an open-ended structured questionnaire and the analysis selected only variables highlighting the main features pertaining to the objective of the study. With the aim of assessing the factors affecting migration decisions at household level, the binomial logistic regression model with a dichotomous dependent variable Y_i with two values, 1 (when a household member has decided to migrate) or 0 (otherwise), data will be analyzed using the binomial logistic regression model (see Hosmer and Lemeshew, 1989). The set *X* of *p* explanatory variables (made of both push and pull characteristics) is made by continuous and categorical/dichotomous variables. The probability that a household *i* has decided to migrate to another area is given by the function: $\pi_i(X) = \frac{e^{\beta_0+\beta_1 X_{i1}+\beta_2 X_{i2}+...+\beta_p X_{ip}}}{1+e^{\beta_0+\beta_1 X_{i1}+\beta_2 X_{i2}+...+\beta_p X_{ip}}}$ (1), and then $\frac{\pi_i}{1-\pi_i}$ is the odds in favor of the household having decided to migrate. Hence, by applying the natural logarithm on both sides of (1) the logit model is then written as:

$\ln\left(\frac{\pi_i}{1-\pi_i}\right) = \beta_0 + \beta_1 X_{i1} + \beta_2 X_{i2} + ... + \beta_p X_{ip}$ (2). Equation (2) is estimated by the maximum likelihood estimation method and the basic assumptions of normality, linearity, and homogeneity of variance for the independent variables are not a requirement.

RESULTS AND DISCUSSIONS

A country's economic status is strongly linked to the transition of its population from one area to another. According to Kainth (2010), uneven economic development, inter-regional disparities and differences in living standards are among the reasons behind migration phenomenon. In this regard, Simpson (2017) argues that if income is highly volatile, workers may be incentivized to search for more stable income streams, especially in countries where credit markets are nonexistent or incomplete. In search of understanding the determinants of migration decision, the literature distinguish two factors of migration, namely, push or pull factor. The *pull* factors constitutes a set of conditions that propel households' members to leave their original living settings, while *push* factors are conditions that entice people to enter a destination country (Simpson, 2017; Ishtiaque and Ullah, 2013; Kainth, 2010; Thet, n.d.). This approach of explaining the driving factors of international migration can easily be applied to inter-regional or intra-country migration analysis. It is based on a basic push-and-pull model (Mansoor and Quillin, 2007) made by economic factors, demographic pressures on land and unemployment status ('push factors') in the sending regions, and natural resources (of which, water and land), higher wages and labor demand, and family reunification ('pull factors') in receiving regions offering better-off livelihoods (Mansoor and Quillin, 2007; Thet, n.d.; with an emphasis of the author). This push-and-pull model will guided the migration decisions analysis conducted in this study especially its parts concerning the push and pull factors by analyzing their level of impact on households' migration decisions. The descriptive analysis of the database under study reveals that Western, Southern and Eastern rural provinces have registered more than 20 percent each in the total internal migrations flows. This let understand that rural areas constitute the sending zones while urban zones are the receiving locations. The Southern province is the one with an increased rate of migrants to others zones with 32.2 percent followed with the Western province with 24.3% and then the Eastern province with 201.2 percent. The reason behind the high rate of migrants in the two first provinces may be the high prevalence of poverty and extreme poverty in those regions. This is line with the findings from statistical analysis which showed that being in a higher socio-economic category is decreasing by 21.5 percent the probability of migrating while being in rural area increase it by 30.5 percent. This empirical finding supports the assertion by FAO (2016) that migration serves as an important strategy for improving rural households' livelihoods as rural people moves to urban areas for wages and other

services. The results show that an increase in one level education is multiplying the likelihood of migrating from home region to another by 1.36 respectively.

Table 1: Binomial Logistic Regression Model: Estimation Results

	Coeff.	St. Err.	t-value	p-value	Sig	Exp (β)
Age	0.011	0.004	3.19	0.001	***	1.011
Sex	0.309	0.086	3.58	0.000	***	1.362
Education	0.208	0.042	4.94	0.000	***	1.231
Household size	0.233	0.023	9.97	0.000	***	1.262
Ratio aged less than 6 years	-0.270	0.353	-0.77	0.444		0.763
Ratio aged 7-15 years	0.092	0.288	0.32	0.749		1.096
Ratio aged 16-60 years	0.893	0.226	3.94	0.000	***	2.442
Agricultural income	0.000	0.000	0.36	0.718		1.000
Land size	0.000	0.000	-1.30	0.193		1.000
Land ownership	-0.272	0.073	-3.72	0.000	***	0.762
Livestock	0.005	0.004	1.33	0.183		1.005
House ownership	-0.036	0.132	-0.28	0.783		0.965
Loan access	0.588	0.070	8.42	0.000	***	1.800
Poverty status	-0.215	0.078	-2.77	0.006	***	0.807
Rural	0.305	0.111	2.74	0.006	***	1.357
Constant	-3.294	0.389	-8.46	0.000	***	0.037

Mean dependent var	0.51	SD dependent var	0.50
Pseudo r-squared	0.08	Number of obs	5035
Chi-square	436.19	Prob > chi2	0.00
Akaike crit. (AIC)	6436.16	Bayesian crit. (BIC)	6586.22

**** p<0.01, ** p<0.05, * p<0.1*

This appears very intuitive and evident for a literate household member as, according to Jiang (2014), high level of education eases internal human capital migration by providing knowledge and skills required in areas full of employment opportunities. In addition, when the household becomes large, adults or those who are able to work will tend to leave the family circle to find out an income generating opportunity to start their own family or become independent. This provides an explanation to the increase in the probability of migrating by the age, the size of the household and the ratio of household members aged between 16 and 60 years increases (see Table 1). Surprisingly, the results reveal that being a female household head multiplies by 1.36 the likelihood of migrating. This finding is in line with the statement by Awumbila (2015) and Awumbila & Ardayfio-Schandorf (2008) that 'migration feminization' is taking place in Ghana. This is not only occurring in Ghana but elsewhere in developing world (Christophe, G. & de

Loenzien, 2014) and particularly in Rwanda as the main reason behind this increase in number of women in migration streams is the high level of poverty in single mothers households (with a non negligible proportion) and female-headed migrants' home families leading them to leave for unskilled occupations such as domestic workers and street vendors (for fruits, clothes, beverages, electronic accessories, …) and bricklayer-assistants mainly available in cities and towns. This trend of female migrants out number male migrants is a recent phenomenon which needs much attention (but out of the scope of this study) to elucidate associated risks and inequalities that affect the female decision's to migrate in sending and receiving areas.

The table 1 also reports that upgrading to a high socio-economic category and land holding decrease the probability migrating. This may be explained by the fact that upgrading to high socio-economic group depend largely on the increase of income and assets endowment at household level. In this case, household members are led to remain in their families and search for ways to value the available assets in their neighborhood.

However, the estimated coefficients on livestock, agriculture income, the land size, the ratio of household members aged less than 6 years and aged between 7 and 15 years, and house ownership are not statistically significant and were not found to be factors affecting the migration decision at household level. These results are not in line with those found in the literature (for example, Gavonel, 2017; Herrera & Sahn, 2013; Peker, 2004). On one hand, this may find its explanation in the high level of school enrolment in rural areas (MINEDUC, 2017), and young people are not concerned by migration issues as they are left at home with their mother or elder siblings when the father or mother leaves for another region in search for livelihoods diversification opportunity. On the other hand, livestock numbers (mainly small livestock) appear to be relatively lower in rural areas and most farmers average less than half a hectare per rural household.

CONCLUSION

This empirical research studied the determinants of migration decisions in rural households of Rwanda. The literature review showed that factors affecting rural households' decision to migrate can be categorized into two main groups: *'push* and *pull factors*'. The results revealed that being a female household head, being an adult from rural areas, an increased number of household size, an advanced level of education are the push factors increasing by around 30% and more the probability of deciding to migrate to another region. On the other hand, owning a land and being reach decreased the likelihood of moving to other zones. This leads to affirm that employment opportunity and availability of diversified livelihoods sources in receiving regions constitute the main pull factors of migration decisions at rural household level. In light of these findings, it is recommended to (I) ensure more balanced regional growth and opportunities for increased access to off-farm employment for a larger proportion of the rural population and (II) carry out a

study on the effects of migration on the livelihoods of migrant-sending households in order to make a thorough and refined situational analysis.

ACKNOWLEDGEMENT

The dissemination of the results of this research would not have been possible without the financial support of the African Center of Excellence in Data Science of the University of Rwanda (ACE-DS/UR).

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Original Scientific paper
10.7251/AGRENG1903076S
UDC 591.1:615.9

MODEL STUDY TO INVESTIGATE THE TOXIC INTERACTION BETWEEN GLYPHOSATE AND COPPER SULPHATE ON CHICKEN EMBRYOS

Rita SZABÓ[1]*, Péter BUDAI[1], Éva KORMOS[1], István BUDA[1], Adrienn GRÚZ[1], Géza SZEMERÉDY[1], József LEHEL[2], Rita FÁTH[1]

[1]University of Pannonia, Georgikon Faculty, Institute of Plant Protection, Hungary
[2]University of Veterinary Medicine, Department of Food Hygiene, Hungary
*Corresponding author: szabo-r@georgikon.hu

ABSTRACT

The toxic effects of the Taifun Forte herbicide (360 g/l glyphosate isopropylamine salt) applied alone or in combination with copper sulphate were studied on chicken embryos in the early phase of embryonic development. The test materials were injected in 0.1 ml volume into the air chamber of eggs on the first day of incubation. Subsequently, on the third day of incubation permanent preparations were made from the embryo in order to study the early developmental stage. Embryos fixed on slides and stained with osmium tetroxide solution were studied under light microscope. The embryonic mortality and the developmental anomalies was analysed statistically by Fisher test. According to the result of the statistical evaluation, the embryonic mortality was not influenced by the single treatment of copper sulphate. However, Taifun Forte and its combination with heavy metal significantly increased the early embryonic mortality. Developmental abnormalities were sporadically observed due to the single administration of copper sulphate. The incidence of it was increased due to the treatment with herbicide alone and in combination with copper sulphate. Based on the results, additive toxic interaction may occur between the copper sulphate and glyphosate that can highly reduce the viability of the embryos or can lead to extinction of wild birds in serious cases.

Keywords: *glyphosate, copper sulphate, interaction, embryonic mortality, chicken embryo.*

INTRODUCTION

The chemical plant protection process is one of the most important polluting activities in the agricultural production. Sprayed pesticides and other xenobiotics, e.g. heavy metals, due to the agricultural activities during the plant protecting processes, can contaminate the ecosystem of a given habitat simultaneously. Therefore, the chemical load can occur as a complex problem, so the combined toxic effect, i.e. toxic interaction of at least two substances can be expected and the components can modify the effect of each other.

For several years, our research team has been conducting animal experiments aimed at determining the embryotoxic and teratogenic effects of pesticides and heavy metals by the use of avian embryos (Budai *et al.*, 2001). In the framework of these studies, the embryotoxic effects of the materials tested were monitored primarily in the late phase of embryonic development. Recently, a new processing technique has been introduced, which makes it possible to evaluate also the early phase of embryonic development. By the use of staining with 0.1% osmium tetroxide it is now possible to make permanent preparations of the embryo in the early phase development (between days 1 and 4) to determine any changes in embryo morphology and viability by light microscopy (Várnagy, 2005).

The objective of this study was to determine the individual and combined embryotoxic effects of heavy metal (copper) modelling the heavy metal load of the environment and an optionally selected pesticide widely applied in the practice (Taifun Forte). As the ecotoxicological test methods used in the practice are mainly limited to study the toxic effect of compounds used alone, data on interactions between pesticides can be regarded as gap-filling information, especially in relation to the avian organism (Thompson, 1996). Furthermore, the interaction effects are examined not only in the field of ecotoxicology, but also in all other areas that deal with health care and chemical safety issues (Oskarsson, 1983; Danielsson *et al.*, 1984; Speijers and Speijers, 2004).

MATERIALS AND METHODS

For modelling the environmental copper load, 0.01% copper sulphate solution (Reanal-Ker Ltd., Hungary) was used in individual and combined treatment. At present, copper is used primarily for wire manufacturing, as a chemical catalyst and for the production of alloys. It is applied as a nutrient in plant cultivation and as a bactericidal, fungicidal and algicidal agent in chemical plant protection. In veterinary medicine copper is used as a feed additive, growth promoter and disease-preventing substance (Adriano, 1986).

The herbicide Taifun Forte (360 g/l glyphosate [isopropylamine salt], Adama Hungary Ltd., Budapest, Hungary) was used in individual and combined treatment in typical field application rate (2.5%). It is a phosphorous-containing pesticide containing 360 g/l glyphosate isopropylamine salt as active ingredient and assigned to marketing category III. It is used widely on arable land as well as in horticulture and viticulture, for perennial and seedlings of single and dicotyledonous plants, for drying and total weed control. The product is not toxic to bees and moderately toxic to fish (NFCSO, 2012).

The study was conducted on purebred fertile Farm hen's eggs derived from the stock farm of Goldavis Ltd. (Sármellék, Hungary). The eggs were incubated in a Ragus type hatcher (Vienna, Austria). During the incubation the appropriate temperature (37–38°C), air humidity (65–75%) and the daily rotation of eggs were provided (Bogenfürst, 2004). The treatment of eggs (n=10/group) was performed on the day of initiation of hatching. In case of individual treatment, solution and/or emulsion made from test chemicals in 0.1–0.1 ml end volume were used while 0.2

ml of the chemical agents were injected into the air chambers of eggs in case of combined application (Clegg, 1964; Várnagy *et al.*, 1996; Kertész, 2001; Palkovics, 2003). For the preparation of solution and/or emulsion as well as in the control treatment, distilled water was used. The incubation was started immediately after the treatments.

In order to study the early phase of development, permanent preparations were made from 10 embryos per group on day 3 of incubation. Above the air chamber the calcic eggshell and the shell membrane were removed, then the germinal disk was cut around and stained with 0.1% osmium tetroxide solution. The stained germinal disk was placed into avian physiological saline solution (0.75 w/v%) with 38^{o}C temperature and it was floated on a slide and fixed with DPX histological adhesive. Finally the slide was covered with coverslip. The permanent preparations were then examined by light microscopy (Sinkovitsné and Benkő, 1993; Kertész, 2001). In case of the biometric processing of the embryonic mortality and developmental anomalies, exact test according to Fisher was used.

RESULTS AND DISCUSSION

Embryonic mortality

On day 3 after treatment, only a single dead embryo (10.0%) was found in the control group (Table 1).

As a result of treatment with copper sulphate, the rate of embryonic mortality was 10.0%. The difference was not significant (Table 1).

The single administration of Taifun Forte herbicide increased the embryonic mortality up to 60.0%. This change was significant ($p<0.05$) as compared to the control group (Table 1).

The combined administration of herbicide and copper sulphate resulted in an embryonic mortality rate of 80.0%. According to the statistical evaluation, the change was statistically significant as compared to both the control group ($p<0.01$) and the group treated with copper sulphate alone ($p<0.01$) (Table 1).

Table 1. Embryonic mortality from teratogenicity test of Taifun Forte and copper sulphate in chicken embryos after single and combined administration

Treatment	**Death No / No fertile eggs**	**Rate of embryonic mortality (%)**
Control	1/10	10.0
Copper sulphate	1/10	10.0
Taifun Forte	6/10 [a1]	60.0
Taifun Forte + Copper sulphate	8/10 [a2, b]	80.0

[a]Significant difference as compared to the control group ([a1]$p<0.05$; [a2]$p<0.01$)

[b]Significant difference as compared to the group treated with copper sulphate alone ($p<0.01$)

Developmental anomalies

During the light-microscopic evaluation of permanent preparations stained with osmium tetroxide, no embryos showing developmental anomalies were found in the control group (Table 2 and 3).

One of the embryos treated with copper sulphate showed developmental anomaly (11.1%). This rate was not significantly different from that found in the control group (Table 2). The developmental anomaly consisted of retarded development of the embryo and its vascular system (Table 3).

Two embryos (50.0%) showed abnormal development as a result of the treatment with Taifun Forte herbicide alone. This change was not significant as compared to the control group (Table 2). The developmental anomaly consisted of retarded development of the embryo and its vascular system (Table 3).

Due to the combined treatment, the rate of developmental anomalies was increased to 50.0%. The change was not significant as compared to both the control group and the groups treated with either Taifun Forte or copper sulphate alone (Table 2). The type of developmental anomaly was retarded development of the embryo and its vascular system (Table 3).

Table 2. Developmental anomalies from teratogenicity test of Taifun Forte and copper sulphate in chicken embryos after single and combined administration

Treatment	**No of embryos showing developmental anomalies / No of live embryos**	**Rate of developmental anomalies (%)**
Control	0/9	0.0
Copper sulphate	1/9	11.1
Taifun Forte	2/4	50.0
Taifun Forte + Copper sulphate	1/2	50.0

Table 3. Types of developmental anomalies diagnosed in the teratogenicity test of Taifun Forte and copper sulphate in chicken embryos after single and combined administration

Treatment	**Types of developmental anomalies (incidences of developmental anomalies)**
Control	No anomaly
Copper sulphate	Poorly developed vasculature (1) Poorly developed body (1)
Taifun Forte	Poorly developed vasculature (2) Poorly developed body (2)
Taifun Forte + Copper sulphate	Poorly developed vasculature (1) Poorly developed body (1)

The results of study on the toxic effects of copper sulphate and glyphosate containing herbicide used alone or in combination in the early phase of embryonic development allow us to draw the following conclusions.

It can be established that the embryonic mortality found in the group treated with copper sulphate alone was not significantly different from that seen in the control group.

The embryonic mortality was higher in the group treated with Taifun Forte herbicide, than in the control or group treated with copper sulphate alone.

At the same time, it can be stated that the combined treatment with copper sulphate and the herbicide clearly resulted in enhanced embryo toxicity, since the rate of embryonic mortality found in the combination treatment group was significantly higher than that obtained in the control group or in the group treated with copper sulphate alone.

This is in harmony with the results of previous studies in which treatments were performed at different times of the incubation period and the eggs were opened and the results evaluated on day 19 of the incubation period. It was concluded that the combined treatment resulted in increased embryotoxic effect in comparison with the individual embryo damaging effect of the used components (Budai *et al.*, 2002).

The intravenous injection of copper salts into pregnant hamsters on day 8 of gestation caused an increase in embryonic resorptions as well as the appearance of developmental malformations in surviving offspring (Ferm and Hanlon, 1974).

Glyphosate containing RoundUp herbicide was examined by other researchers in Wistar rats. Rat dams were treated orally with 500, 750 and 1000 mg/kg glyphosate via drinking water. Results showed a 50% mortality rate for dams treated with 1000 mg/kg glyphosate. Skeletal alterations were observed in all treated groups. Based on the data the authors concluded that the glyphosate containing RoundUp is toxic to rat dams and induces developmental retardation of the fetal skeleton (Dallegrave *et al.*, 2003).

In view of the increased sensitivity of wild fowl species, the study reported in this paper should be extended to seed-eating birds (pheasants, Japanese quail) and waterfowl (mallards). We also recommend that the interaction studies should be complemented with hatchability studies and investigations performed at the postembryonic stage of development, so that the harmful effects of the chemicals under study can be explored more precisely.

CONCLUSIONS

Based on the results of our avian teratological study performed by applying of Taifun Forte (in typical field rate) and copper sulphate (inducing relatively low environmental copper load which could be less embryo toxic in itself) individually and simultaneously, it was established that joint effects of both chemical agents additively increased the embryonic mortality under the circumstances used in our experiments. According to the published literature, the join toxic effect of many pesticide combinations is at least additive. In some cases, pesticide mixtures, if

they particularly contain insecticide component, have been shown to be synergistic, with reported increase in toxicity up to 100-fold. However, these effects are species, time and dose dependent and are therefore difficult to predict routinely (Thompson, 1996).

ACKNOWLEDGEMENT

The publication was supported by the EFOP-3.6.3-VEKOP-16-2017-00008 project. The project was co-financed by the European Union and the European Social Fund.

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Original Scientific paper
10.7251/AGRENG1903083J
UDC 637.1:632.95(548.7)

PESTICIDE RESIDUES IN COW MILK AND DAIRY PRODUCTS FROM THE MAJOR MILK PRODUCING AREA OF SRI LANKA

Jagath JAYASINGHE[1*], Samudra PATHIRANA[1], Dhammi DILHANI[1], Senevirathne NAVARATHNA[1], Manoj SINHAPURA[2], Chamila JAYASINGHE[3], Rohana CHANDRAJITH[4], Upul MARAPANA[1]

[1]Department of Food Science and Technology, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda, Sri Lanka
[2]Department of Chemistry, Faculty of Applied Sciences, University of Sri Jayewardenepura, Nugegoda, Sri Lanka
[3]Department of Food Science and Technology, Faculty of Livestock, Fisheries and Nutrition, Wayamba University of Sri Lanka, Makandura, Sri Lanka
[4]Department of Geology, Faculty of Science, University of Peradeniya, Peradeniya, Sri Lanka
*Corresponding author: jagathj@sci.sjp.ac.lk

ABSTRACT

Nuwara Eliya district is the leading fresh milk producing area in Sri Lanka. In the district, pesticides are widely applied for intensive cultivation of vegetables which leads to contamination of water and material used to feed cows. Contamination and health risk hazards of organophosphorus pesticide residues in milk and dairy products originated in the district were studied. Identification and quantification of eleven commonly used pesticides in 50 milk samples and 12 dairy product samples were performed using standard analytical methods and GC-MS technique. Results revealed that fresh milk contained residues of Prothiofos (0.0568±0.037 mg/kg), Diazinon (0.0378±0.009 mg/kg), Chlorpyrifos (0.0264±0.004 mg/kg), Profenofos (0.196±0.099 mg/kg), Fipronil (0.1906±0.188 mg/kg), Phenthoate (0.1012±0.110 mgkg), Dimethoate (0.1196±0.201 mg/kg) and Tebuconazole (0.062±0.069 mg/kg) at higher levels than the recommended maximum residue levels (MRLs) of the World Health Organization. Sterilized milk (0.0115±0.000 mg/kg) and fermented milk (0.022±0.004 mg/kg) contained higher levels of Profenofos than the MRLs. Higher levels of Fipronil than MRLs were observed in pasteurized milk (0.086±0 mg/kg) and fermented milk (0.014±0.000 mg/kg) samples. Phenthoate at higher levels than MRL was reported in pasteurized milk (0.3645±0.402 mg/kg), sterilized milk (0.1405±0.197 mg/kg) and milk powder (0.0055±0.000 mg/kg). Moreover, Dimethoate content in fermented milk (0.087±0.012 mg/kg) was higher than the MRL. Routine monitoring of the above pollutants in food items including fresh milk and value added milk products is essential to prevent, control and reduce the pollution and to minimize the health risks to consumers.

Key words: *Pesticide residues, Cow milk, Dairy products, Health risk*

INTRODUCTION

Organophosphorus pesticides have been extensively used in Sri Lanka to increase crop production. Especially, farmers in the central part of the island who cultivate upcountry (elevation >900 m of sea level) vegetables continuously using intensive cultural practices with hybrid varieties used to apply pesticides beyond the manufacturers recommended levels (Dilhani *et al.*, 2015). Intensive usage of pesticides has resulted in trace contamination of air, water and soil with their residues (Pandit *et al.*, 2002). The residues of these pesticides can be absorbed by milk producing animals such as cows through contaminated feed, water and inhaled air (Pandit *et al.*, 2002; Ravichandran *et al.*, 2015). Milk is the most versatile organic food product of animal origin (Ghidini *et al.*, 2005). Pesticide residues being highly lipophilic are primarily stored in fatty tissues in cows and later excreted through milk fat. As a result, consumers are at a risk of exposing to these pesticide residues as they are accumulated in fresh milk and fat rich dairy products (Nigam and Siddiqui, 2001; Ravichandran *et al.*, 2015). Most organophosphorus pesticides undergo degradation by hydrolysis, yielding nontoxic, water soluble products. Therefore, toxic hazards of organophosphorus pesticides are short term, however, they show higher acute toxicities (Darko and Akoto, 2008). The inhibition of acetylcholinesterase in the nervous system, resulting in respiratory, myocardial and neuromuscular transmission impairment is the toxicological effect of the organophosphorus pesticides (Goh *et al.*, 1990). Therefore, pesticide residues in food represent a significant health risk (Darko and Akoto, 2008).

Nuwara Eliya district, which is located in the central area of the island, is producing the highest amount of fresh milk (72 Mn l/yr) in Sri Lanka (Department of Animal Production and Health, 2015). A large number of milk farmers sell fresh milk to processors in the district for manufacturing dairy products. It is a common practice that milk farmers in the Nuwara Eliya district use crop residues and grass from the surrounding area to feed their cows (Dilhani *et al.*, 2015). Moreover, they use surface and ground water sources to feed cows which could be easily contaminated with the intensive application of pesticides for vegetables (Pathirana *et al.*, 2015). The effect of regular intake of pesticide residues in food is hard to detect and quantify. However, management and regulation of these chemicals are vital considering the quality of milk and the risks associated with human health. This study therefore, seeks to provide the baseline information on the contamination levels of organophosphorus pesticide residues in fresh milk and dairy products originated in the main fresh milk producing area of Sri Lanka.

MATERIALS AND METHODS

Chemicals and reagents

Pure and mixture of standards were purchased from Dr. Ehrenstofer Co. (Augsburg, Germany). These certified pesticide standards contained greater than 98% purity. Internal standard, Triphenyl phosphate was purchased from

AccuStandard, USA. Florisil (60-100 mesh) was purchased from Fluka Analytical. All other solvents and reagents were purchased from Sigma Aldrich.

Milk and dairy products sampling

The list of milk collecting centers belonged to the government and private sector located in the Nuwara Eliya district was obtained from the District Veterinary Office, Nuwara Eliya. Fresh milk samples were taken from milk chilling tanks of 62 randomly selected milk collecting centers. Samples were collected in to clean and sterile amber colored plastic bottles. The milk samples were kept on ice immediately after collection and transferred to the Laboratory and stored at -4^0C till the time of analysis. Four types of dairy products namely pasteurized milk (UHT milk), sterilized milk (Bottled milk), fermented milk (Yoghurt) and spray dried milk (Full cream milk powder) originated from Nuwara Eliya district were collected from randomly selected retail outlets located in the district. These samples were stored at 4-6^0C before subject to further analysis.

Preparation of standard solutions

Stock standard solutions of pesticides were prepared at a concentration of 500 mg/l in acetone and methanol, depending on the compound's solubility. Stock solution of internal standard, Triphenyl phosphate was prepared at a concentration of 500 mg/l. A working standard solution of pesticides and internal standard at the concentrations of 5 mg/l were prepared. The stock and working standard solutions were stored at 4^0C until needed.

Quality control and quality assurance

To determine the method quality the linearity, recoveries, limits of detection (LOD) and limit of quantification (LOQ) were tested. The limits of detection and of quantification of each substance were determined initially with standard solutions. LOD was calculated as three times the signal-to-noise ratio. LOQ was calculated as 10 times the signal-to-noise ratio.

Extraction and cleanup of milk samples

A chromatographic tube was filled with 100 ml petroleum ether and 25 g of standardized Florisil was slowly added. The adsorbent was allowed to settle, and petroleum ether was drained to a level of about 50 ml above the top of the adsorbent. A total of 25 g Florisil was added in small portions to the milk sample (10 g). While adding the Florisil, milk sample was stirred continuously with a glass rod until a homogenous, free-flowing powder was obtained. Then resulted powder was packed to the column, washed with petroleum ether and the same was collected in a 1-1 round-bottomed flask. Then the column was eluted with 300 ml of the eluting mixture at a flow rate not exceeding 5 ml/min, and collected elute in the same flask. Elute was rotary-evaporated and concentrated to about 5 ml. Last traces of solvent were removed with the aid of a gentle stream of air. The remaining residue after evaporation was transferred with a small volume of

petroleum ether into a 5 ml volumetric flask and diluted to the mark. Thereafter, the sample was transferred to a gas chromatography vial and stored in refrigerated condition until use for analysis.

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GC-MS analysis of milk and milk product samples

Gas chromatography- mass spectrophotometry analysis (GC-MS) of fresh milk and milk products for pesticides residues were carried out according to the DFG S9 multi-residue method (Their and Kirchhoff, 1987) using an Agilent Technologies Model 7890A (GC) and Agilent Technologies (MS-5975 C) equipped with a capillary column (HP 5 MS, non-polar inert, 30 m ×0.25 mm diameter×0.25µm film thickness) and an inert XLEI/ CI MSD triple axis detector.

A 1µl aliquot was injected into the GC- MS. The injector was operated in the splitless mode. The column was programmed to operate from 80-160 ^{0}C (10 ^{0}C/min, hold 1 min), 160-250 ^{0}C (6 ^{0}C/min, hold 1 min) and 250-300 ^{0}C (10 ^{0}C/min, hold 5 min). The temperatures of MS Heater were 230-250 ^{0}C for MS source and 150-200 ^{0}C for MS quad. The carrier gas was Helium with a run time of 36 min.

Statistical analysis

Summary statistics (mean and standard deviation) were computed using Minitab 17.0 statistical software.

RESULTS AND DISCUSSION

Method validation

The fresh milk samples and dairy products were analyzed for the presence of Prothiofos, Diazinon, Chlorpyrifos, Oxyfluorfen, Profenofos, Chlorothalonil, Fipronil, Penthoate, Dimethoate, Tebuconazole and Deltamethrin using GC-MS. Method validation is the process of proving that an analytical method is acceptable for its intended purpose. Of the eleven pesticides analyzed, seven were linear across the calibration range with the correlation coefficient of above 0.99. Mean recovery range of milk samples were varied from 80.2 to 100.1% with a relative standard deviation ranged from 7.2-29.1%, indicating an acceptable level of recovery of pesticide residues. Mean LOD and LOQ values were 0.00094 and 0.00290 respectively (Table 1).

Table 1: LOD and LOQ values for pesticides

Pesticide	**LOD (ppm)**	**LOQ (ppm)**
Dimethoate	0.003992	0.013307
Chlorothalonil	0.003600	0.012100
Phenthoate	0.000852	0.002840
Profenafos	0.000623	0.002076
Diazinon	0.000020	0.000060

Oxyfluorfen	0.000153	0.000510
Deltamethrin	0.000105	0.000350
Tebuconazole	0.000251	0.000837
Fipronil	0.000796	0.000238
Chlorpyrifos	0.000030	0.000090
Prothiofos	0.000015	0.000050

Pesticide residue levels detected in milk and dairy products

The results obtained from the analysis of 62 fresh cow's milk and dairy products for selected pesticide residues are shown in Table 2.

Table 2: Pesticide residues present in fresh milk and dairy products samples (mg/kg)

Pesticide	Fresh milk	Pasteurized milk	Sterilized milk	Fermented milk	Milk powder
Prothiofos	0.0568± 0.037	0.0225±0	0.016±0	0.012±0	0.005±0
Diazinon	0.0378±0.009	0.0155±0.004	0.0135±0.0007	0.018±0.001	0.0155±0.003
Chlorpyrifos	0.0264±0.004	0.012±0	0.0095±0.0007	0.0085±0.0007	0.014±0.003
Oxyfluorfen	0.0239±0.004	0.0225±0.045	0.0305±0.028	0.01±0.004	0.0065±0.002
Profenofos	0.196±0.099	0.003±0	0.0115±0	0.022±0.004	0.0065±0
Chlorothalonil	0.0652±0.083	0.05±0	0.0475±0	0.014±0	≤ LOD
Fipronil	0.1906±0.188	0.086±0	≤ LOD	0.014±0	0.018±0
Phenthoate	0.1012±0.110	0.0364±0.402	0.0140±0.197	≤ LOD	0.0055±0
Dimethoate	0.1196±0.201	0.0205±0.013	≤ LOD	0.087±0.012	0.0005±0
Tebuconazole	0.062±0.069	≤ LOD	≤ LOD	0.0045±0	0.006±
Deltamethrin	≤ LOD	0.0035±0	≤ LOD	0.0185±0	≤ LOD

Table 2 shows that except Deltamethrin, all other pesticides were identified as residues in fresh milk. Residue levels of Prothiofos (0.0568 mg/kg; MRL=0.05 mg/kg), Diazinon (0.0378 mg/kg; MRL=0.02 mg/kg), Chlorpyrifos (0.0264; MRL=0.02 mg/kg, Profenofos (0.196 mg/kg; MRL=0.01 mg/kg), Fipronil (0.1906 mg/kg; MRL=0.008 mg/kg), Penthoate (0.1012 mg/kg; MRL=0.003 mg/kg), Dimethoate (0.11961 mg/kg; MRL=0.05 mg/kg) and Tebuconazole (0.062 mg/kg; MRL=0.01 mg/kg) present in fresh milk have exceeded the recommended levels set by the WHO (World Health Organization, 2006). Among the pesticides Profenofos (0.196±0.099 mg/kg; MRL=0.01 mg/kg) reported the highest level of residues in fresh cow's milk. The levels of Oxyfluorfen and Chlorothalonil residues observed were below the MRLs of WHO. A similar study in India has identified monocrotophous in the fresh milk samples (Ravichandran *et al.*, 2015). Organophosphorus pesticide residues have been detected in cow's and buffalo's milk products (John *et al.*, 2001). Milk samples analyzed for contaminants revealed that Chlorpyrifos and Diazinon levels presence in the samples were (0.278 mg/kg

and 0.220 mg/kg, respectively) higher than the acceptable limits published by WHO (World Health Organization, 2006). Moreover, the presence of organophosphorus pesticide residues in vegetables has been reported by several researchers; in the Egyptian market (Dogheim *et al.*, 2002) in China (Bai *et al.*, 2006) and in Ghana (Darko and Akoto, 2008).

In general, value added milk products indicated lower levels of pesticide residues compared with the levels detected in fresh milk. Fipronil reported the highest level of residue in pasteurized milk samples (0.086 mg/kg; MRL=0.003 mg/kg) which is higher than the MRL set by WHO. All other pesticide residues present in pasteurized milk had lesser residue levels compared to MRLs of WHO.

Analysis of sterilized milk samples showed that residue levels of Profenofos (0.0115 mg/kg), Chlorothalonil (0.0475 mg/kg) and Phenthoate (0.0140 mg/kg) have exceeded the MRLs of 0.01 mg/kg, 0.02 mg/kg and 0.003 mg/kg respectively. The levels of Prothiofos, Diazinon, Chlorpyrifos and Oxyfluorfen residues were lesser than MRLs of WHO. Moreover, Fipronil, Dimethoate, Tebuconazole and Deltamethrin were below the level of detection in all sterilized milk samples analyzed. It shows that high pressure and temperature used in the manufacturing process (sterilization) has destroyed the residues of Fipronil, Dimethoate and Tebuconazole present in fresh milk.

Fermented milk samples were contaminated with Profenofos (0.0115 mg/kg), Fipronil (0.014 mg/kg) and Dimethoate (0.087 mg/kg) and these values are above the MRLs of 0.01 mg/kg, 0.008 mg/kg and 0.05 mg/kg respectively. The product contained other pesticides at lesser levels than MRLs. However, Penthoate which was present in fresh milk was not detected in fermented dairy products.

Milk powder samples contained two pesticides namely Fipronil (0.01 mg/kg) and Phenthoate (0.0055 mg/kg) at higher levels than MRLs of 0.008 mg/kg and 0.003 mg/kg respectively. Chlorothalonil and Deltamethrin were not detected in milk powder samples. Chlorothalonil has been destroyed by the powder manufacturing process of milk. Other pesticide residues were observed at very low levels in milk powder than MRLs set by WHO.

The source of contaminants could be most probably the feed material used by farmers. In the Nuwara Eliya district, milk farmers feed cows with grasses, crop residues and water which were contaminated due to an extensive use of pesticides in the area (Dilhani *et al.*, 2015, Pathiran *et al.*, 2015). It was observed that farmers use semi intensive (11%) and extensive (15%) raring methods in Nuwara Eliya district (Pathirana *et al.*, 2015). Feed and grasses offered to animals are often contaminated with pesticide residues and after feeding, these residues pass through the body systems (Prassad *et al.*, 2001). When plant materials contaminated with pesticide residues are eaten by herbivores, they are transferred to the food chain, through the main animal originated food sources like milk and meat (Bulut *et al.*, 2011). Moreover, other factors may also contribute to a different degree of contamination that include the application of pesticides on farm animals, environmental contamination and accidental spills (Goordazi *et al.*, 2010).

CONCLUSION

The present study analyzed fresh milk and dairy products collected from the Nuwara Eliya district in Sri Lanka using GC-MS to examine the presence of organophosphorus pesticide residues. Fresh milk contained residues of Prothiofos, Diazinon, Chlorpyrifos, Profenofos, Fipronil, Phenthoate, Dimethoate and Tebuconazole at higher levels than the recommended MRLs of WHO. Compared to other pesticide residues the amount of Fipronil reported the highest residue levels in fresh milk, pasteurized milk and milk powder. Of the sterilized milk samples the highest residue level was reported by Chlorothalonil followed by Oxyfluorfen. Dimethoate content available in fermented milk was the highest among other pesticides. Except for Fipronil, residues of other pesticides present in milk powder showed lower values than MRL. Considering the present level of contamination, preventing the contamination of feedstuffs and ground water sources by pesticides and monitoring the pesticide residue levels of fresh milk are vital to minimize the health risks.

ACKNOWLEDGEMENT

Authors acknowledge the financial support provided by the National Research Council of Sri Lanka under the Grant No. 13-008.

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Original Scientific paper
10.7251/AGRENG1903091A
UDC 632.4

MOLECULAR PHYLOGENY OF *FUSARIUM OXYSPORUM* SPECIES COMPLEX ISOLATED FROM EGGPLANT AND PEPPER IN TURKEY

Hacer Handan ALTINOK[1*] Muhammed Bahattin TANYOLAÇ[2], Duygu ATEŞ[2], Canan CAN[3], Hilal ÖZKILINÇ[4]

1Erciyes University, Agriculture Faculty, Department of Plant Protection, 38039, Kayseri, Turkey
2Ege University,Department of Bioengineering,Bornova-İzmir 35100, Turkey
3Gaziantep University, Faculty of Science, Department of Biology, 27310 Gaziantep, Turkey
4Çanakkale Onsekiz Mart Department of Molecular Biology and Genetics, 17100, Çanakkale, Turkey
*Corresponding author: ahandan@gmail.com

ABSTRACT

Members of *Fusarium oxysporum* species complex (FOSC) are economically most important plant pathogenic fungi found in a large number of plants in different families, while individual strains have strong specificities for particular hosts. Fusarium wilt caused by *F. oxysporum* f. sp. *melongenae* (Fomg) and *F. oxysporum* f. sp. *capsici* (Foc) is a worldwide soil-borne disease that causes yield losses in eggplant and pepper (Solanaceae) growing regions of Turkey. The intra-species or inter-species genetic diversity and phylogenetic relationships in Fomg and Foc isolates obtained from Turkey were investigated by utilizing the sequence data obtained through the designed primers belonging to three protein coding gene regions; beta-tubulin (BT), calmodulin (CAL) and chitin synthase (CHS). Phylogenetic analyses were carried out with BT, CAL and CHS gene regions with a selected subset of Fomg and Foc, along with other non-host *F. oxysporum* and outgroup isolates. The clustering trees successfully separated the Fomg and Foc from the outgroup *Fusarium* isolates. On the other hand, sequence data for BT, CAL and CHS gene regions displayed limited variation among Fomg and Foc isolates and found inefficient to distinguish reliably among several *F. oxysporum* forma specialis (f. sp.) groups. The sequence variation on these 'housekeeping gene' regions in the core genome was not considered adequate to differentiate FOSC populations.

Keywords: *Fusarium wilt, phylogenetic analysis, DNA sequence*

INTRODUCTION

The pathogenic *Fusarium oxysporum* species complex (FOSC) causes major yield losses in the family Solanaceae including on tomato, pepper and eggplant productions in Aegean and Mediterranean Regions of Turkey where the vegetable growing is concentrated. *Fusarium oxysporum* is a ubiquitous asexual species of soil-borne plant pathogens and responsible for vascular wilt or root rot disease in a variety of host plants. These pathogenic strains have been grouped into formae speciales (ff. spp.) and races based on plant and cultivar specificity (Armstrong and Armstrong, 1981). Fusarium wilt caused by *F. oxysporum* f. sp. *melongenae*; Fomg) is one of the most destructive and widely distributed pathogen of eggplant (*Solanum melongena* L.) in Turkey (Altinok, 2005). Fusarium wilt is caused by *Fusarium oxysporum* (Schlect.) emend. Synd. and Hans. f. sp. *capsici* Riv. (Foc), which and it has recently emerged in local pepper fields in Turkey. The characteristic symptoms both Fusarium wilt diseases include wilting, stunting, leaf chlorosis, interveinal yellowing of the outer leaflets, then vascular necrosis and finally death of the above ground parts of the plant (Black and Rivelli, 1990; Agrios, 2005; Altinok, 2005). These pathogens may remain viable for a long period of time in soil as chlamydospores, even after rotation with non-host crops (Nelson *et al.*, 1983; Katan, 1999; Altinok *et al.*, 2018). Currently the preferred and efficient method for control of soil-borne fungal diseases is to take advantage of resistant cultivars. Resistant commercial cultivars yet to be developed, however, some grafted plants are presenting a good level of resistance against Fomg (Gisbert *et al.*, 2011; Altinok *et al.*, 2014). Conventional control of FOSC is inadequate in most cases. DNA-based diagnostic tools allow genetic diversity and phylogeny of *Fusarium* species. Many methods have been used to characterize the genetic diversity within and among populations and evolutionary origin of FOSC, including restriction fragment length polymorphisms (RFLPs), random-amplified polymorphic DNA (RAPDs), amplified fragment length polymorphisms (AFLPs), simple sequence repeats (SSRs) and comparisons of DNA sequences from conserved genomic regions (Assigbetse *et al.*, 1994; Appel and Gordon, 1995; Jarne and Lagoda, 1996; O'Donnell *et al.*, 1998; Baayen *et al.*, 2000). Sequence-based identification of fungi, namely, DNA barcoding using the ITS region and protein coding regions of nuclear DNA by sequence alignments have also been used extensively in fungi phylogenetic relationships. The most commonly used sequence-based markers in *F. oxysporum* include 'housekeeping gene'. Sequence analysis of the nuclear ribosomal DNA (rDNA), and nuclear rDNA intergenic spacer (IGS), translation elongation factor 1-α (TEF-1α), β-tubulin, histone, actin, chitin synthase, and calmodulin genes, have been used to determine the genus *Fusarium* (Jiménez-Gasco *et al.*, 2002; Schmitt *et al.*, 2009). TEF-1α and actin genes are able to detect polymorphisms of the DNA sequence of a genome and involved in basic cellular functions encode highly conserved proteins identified in fungi (Schmitt *et al.*, 2009; Altinok *et al.*, 2018).

In this study, intra-species or inter-species genetic diversity and phylogenetic relationships in Fomg and Foc isolates obtained from Turkey were investigated by

utilizing the sequence data obtained through the designed primers belonging to three protein coding gene regions; beta-tubulin (BT), calmodulin (CAL) and chitin synthase (CHS) gene sequences analyzed with the hierarchical maximum likelihood clustering method.

MATERIALS AND METHODS

Fungal isolates A subset of 40 Fomg and 40 Foc isolates, along with non-host f. sp. isolates (other FOSC) were selected for phylogenetic analyses based on beta tubulin (BT), calmodulin (CAL) and chitin synthase (CHS) sequences. Designed primers (Operon Technologies GmbH, Cologne, Germany). were given in Table 1. All isolates were obtained from the culture collection of Mycology Laboratory of Plant Protection Department of Erciyes University, Turkey (Project No: TUBITAK-TOVAG-109O524 and TUBITAK-COST-114O866).

DNA extraction, PCR amplification, sequencing and phylogenetic analysis Monoconidial Fomg and Foc isolates representing various geographical locations were selected as a subset. For DNA extraction, the method described by Altinok *et al*. (2018) was used. The PCR reactions were prepared in a total volume of 20 µL containing 10x PCR buffer, 2 mM of dNTPs, 10 mM of each primer, 1 U of Taq DNA Polymerase and genomic DNA (25 ng). A negative control with double-distilled sterile water instead of DNA template was used. PCR conditions; started with 5 min of denaturation at 94°C; followed by 35 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C; and a final extension for 10 min at 72°C. Amplification products were electrophoresed on agarose gel in 0.5X Tris-borate EDTA buffer, and stained with the fluorescent dye ethidium bromide and visualized (DNR Bio-Imaging System) under UV light. PCR products were sequenced by Ref-Gen Biotechnology (Ankara, Turkey). BT, CAL and CHS consensus sequences were generated using Geneious software (v10.0.5) (Biomatters, NZ, USA). Multiple alignments of consensus sequences were made with ClustalW (Larkin, 2007). The Megablast algorithm was used to perform similarity (Hall, 1999; Morgulis *et al.*, 2008). Dendrogram was created with MEGA 7 using UPGMA method, with 2000 bootstrap repetitions (Sneath and Sokal, 1973; Huelsenbeck and Ronquist, 2001).

Table 1. Primer codes and sequences used for the analysis of the beta tubulin, calmodulin and chitin synthase genes of the *Fusarium oxysporum* f. sp. *melongenae* and the *Fusarium oxysporum* f. sp. *capsici* in this study.

Primer	**Code**	**Sequence 5´ to 3´**
Beta-tubulin*	Bt-F	5'-CAA GAA CTC AGA GTA TTT CGT C-3'
	Bt-R	5'-TAT GAA CTA GAA GGG GAT GAA G-3'
Calmodulin*	CAL-F	5'-CTC ACT TAC TGA AGA GCA AGT CT-3'
	CAL-R	5'-TTA GTA CTG ACC GTC CTC TAA TC-3'
Chitin synthase*	CHS-F	5'-CTA CAT TCG CTC CTA CCT ACT C-3'
	CHS-R	5'-TGGAAGAACCATCTGTGAGAGTTG-3'

*Primers were designed from the full genome *Fusarium oxysporum* within the scope of our project (TUBITAK-COST 114O866).

RESULT AND DISCUSSION

The Fomg and Foc isolates yielded a single amplicon with a size of 490-500 bp for BT, CAL and CHS in conventional PCR. Amplification products obtained by chitin synthase primer of Fomg (P) and Foc (B) isolates on agarose gel were presented in Figure 1. Geographical origin of Fomg and Foc isolates recovered from greenhouses/fields in Turkey is presented in Table 2. Phylogenetic analyses were carried out using BT, CAL and CHS protein coding gene regions with a selected subset of Fomg and Foc, along with other non-host *F. oxysporum* (*F. oxysporum* f. sp. *lycopersici*-Fol, *F. oxysporum* f. sp. *radicis-lycopersici*-Forl and *F. oxysporum* f. sp. *melongenae*-Fomg) and outgroup *Fusarium* isolates from GenBank (Foc-BT tree *F. solani*-AM419425.1. and *F. incarnatum*-JN614905.1, Fomg-BT tree *F. incarnatum*-JN614905.1 and *F. sublunatum*-KM232076.1, Foc/Fomg-CAL tree *F. proliferatum*-AJ560773.1 and *F.incarnatum*-HQ412341.1, Foc/Fomg CHS tree *F. culmorum*-KP195141.1 and *F. graminearum* AJ314860.1).

The BT, CAL and CHS gene region sequences were yielded and clustering trees successfully separated the FOSC (Fomg, Foc, Fol and Forl) from the outgroup *Fusarium* isolates. One of the cluster contained the whole subset of FOSC, while the other clusters contained the outgroup *Fusarium* isolates. The dendrograms (cluster tree) exhibited mostly similar branching, for that reason Foc-CAL tree is represented as examples in Figure 2. The BT, CAL and CHS gene region was found to be inadequate to provide an intraspecific differentiation for the FOSC. All of the FOSC were grouped together and there was no significant and meaningful discrimination among non-host *F. oxysporum* isolates (Fol and Forl). On the other hand, sequence data for these gene regions displayed limited variation among Fomg and Foc isolates. The sequence similarity for these gene regions was not related to the virulence or geographical origin of the Fomg and Foc isolates. The sequence variation on these 'housekeeping gene' regions in the core genome were not considered adequate to differentiate FOSC populations. Host-specificity is associated with the presence of accessory or lineage-specific (LS) chromosomes and pathogenicity chromosomes are composed of 'transposable elements'. These chromosomes contain effector genes associated with pathogenicity. Pathogenic effector proteins are important proteins for virulence, and these proteins interfere with host proteins (De Wit *et al.,* 2009). Horizontal transfer of accessory chromosomes seems to act as a powerful force on pathogen populations resulting in evolution of new virulent pathotypes (Ma *et al.,* 2010). For that reason the FOSC shares highly similar core chromosomes. The uniform genetic structure of the Fomg and Foc population may be considered as an advantage in plant breeding studies to be carried out to generate resistant pepper cultivars. Basic factors of detection and genetic differentiation of plant pathogens are reported to be virulence ability, host-pathogen relationship, presence of alternative hosts, saprophytic capability and/or long-term resistance to unsuitable environmental (Parker and Gilbert, 2004). In general, plant varieties have two types of resistance; monogenic (vertical), controlled by a single resistance (R) gene and oligogenic (horizontal), controlled by multiple genes. Monogenic resistance has been more effective than

polygenic resistance in other crops and practical to use in modern plant breeding (Beckman and Roberts, 1995; Agrios, 2005).

Table 2. Geographical origin of *Fusarium oxysporum* f. sp. *melongenae* (Fomg) and *Fusarium oxysporum* f. sp. capsici (Foc) isolates collected from greenhouses and fields.

Number	Isolates	Sampling location	Number	Isolates	Sampling location
P1*	HSM-17	Hatay-Samandag	B*1	MSGM-94/1	Manisa-Gölmarmara
P2	Fomg109	Izmir-Menemen	B2	IZTB-80/D-1	Izmir-Torbalı
P3	Fomg102	Izmir-Odemis	B3	ANMK-43/3	Antalya-Kumluca
P4	UBR-9	S.Urfa-Birecik	B4	ANKS-49/8	Antalya-Kas-Demre
P5	BRK-42	Bursa-M.Kemalpasa	B5	MSGM-95/10	Manisa-Gölmarmara
P6	BMK-38	Bursa-M.Kemalpasa	B6	MGML-56/5	Mugla-Milas
P7	DBS-22	Diyarbakir-Bismil	B7	ANSR-42/2	Antalya-Serik
P8	Fomg220	Izmir-Odemis	B8	ANKS-49/2	Antalya-Kas-Demre
P9	ANC-9	Aydin-Incirliova	B9	FOC-2	Mersin-Tarsus
P10	Fomg97	Manisa-Merkez	B10	ANSR-40/2	Antalya-Serik
P11	Fomg122	Izmir-Bayindir	B11	ANGP-21/2	Antalya-Gazipaşa
P12	MS-6	Manisa-Salihli	B12	MRBZ-1/6	Mersin-Bozyazı
P13	ANZ-36	Aydin-Nazilli	B13	MSGM-94/6	Manisa-Gölmarmara
P14	Fomg154	Mersin-Tarsus	B14	ANSR-39/3	Antalya-Serik
P15	Fomg94	Antalya-Serik	B15	ANSR-39/5	Antalya-Serik
P16	Fomg160	Mersin-Tarsus	B16	ANLY-29/4	Antalya-Alanya
P17	UMR-12	S.Urfa-Merkez	B20	ANKS-49-11	Antalya-Kas-Demre
P18	MF-26	Mugla-Fethiye	B21	AYBZ-65-12	Aydın-Bozdoğan
P19	ML-5	Mugla-Milas	B22	IZTB-81/6	Izmir-Torbali
P20	MS-1	Manisa-Salihli	B23	ANSR-42/1	Antalya-Serik
P21	MRM-21	Mersin-Merkez	B24	ANLY-26/1	Antalya-Alanya
P22	ANC-11	Aydin-Incirliova	B25	AYNZ-60/4	Aydin-Nazilli
P24	ML-17	Mugla-Milas	B26	ANMK-43/2	Antalya-Kumluca
P25	MS-9	Manisa-Salihli	B27	IZMN-76/1	Izmir-Menderes
P26	ANZ-40	Aydin-Nazilli	B28	AYBZ-70/5	Aydin-Bozdogan
P27	UMR-28	S.Urfa-Birecik	B29	AYNZ-60/5	Aydin-Nazilli
P28	HSM-21	Hatay-Samandag	B30	AYNZ-60/7	Aydin-Nazilli
P29	ANC-6	Aydin-Incirliova	B31	AYNZ-60/3	Aydin-Nazilli
P30	MY-19	Mugla-Yatagan	B32	AYBZ-65/7	Aydın-Bozdogan
P31	BMK-50	Bursa-M.Kemalpasa	B33	MSSR-85/12	Manisa-Saruhanli
P35	BMK-32	Bursa-M.Kemalpasa	B34	ANGP-18/2	Antalya-Gazipasa
P36	HD-7	Hatay-Dortyol	B35	ANGP-18/A-1	Antalya-Gazipasa
P37	HSK-14	Hatay-Iskenderun	B36	MSSR-86/7	Manisa-Saruhanlı
P38	SCR-45	Samsun-Carsamba	B37	MRBZ-1/9	Mersin-Bozyazi
P39	DBS-21	Diyarbakir-Bismil	B38	IZMN-76/5	Izmir-Menderes
P40	HSK-14	Hatay-Iskenderun	B39	ANLY-29/6-B	Antalya-Alanya
P42	MRM-25	Mersin-Merkez	B40	ANLY-29/6-A	Antalya-Alanya
P43	BRO-55	Bursa-Orhangazi	B41	ANGP-15/1	Antalya-Gazipasa
P44	Fomg172	Izmir-Menemen	B42	ANKS-50/3	Antalya-Kas-Demre
P45	HSM-29	Hatay-Samandag	B43	MGML-65/5	Mugla-Milas

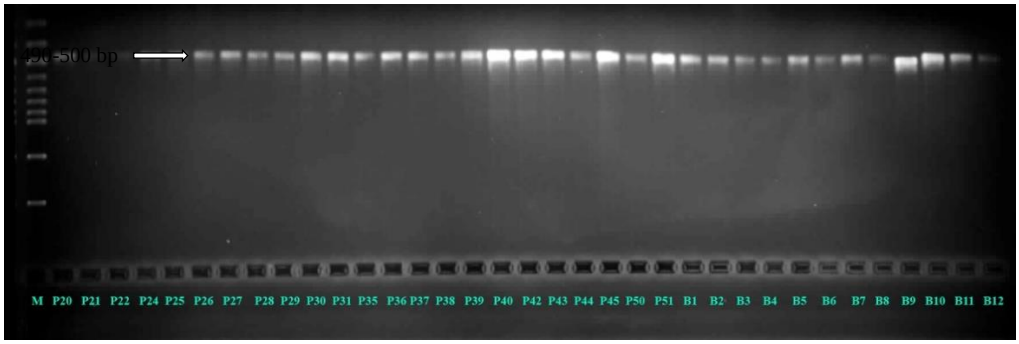

Figure 1. PCR products obtained by chitin synthase primer of Fomg (P) and Foc (B) isolates on agarose gel. Marker (M); 100 bp.

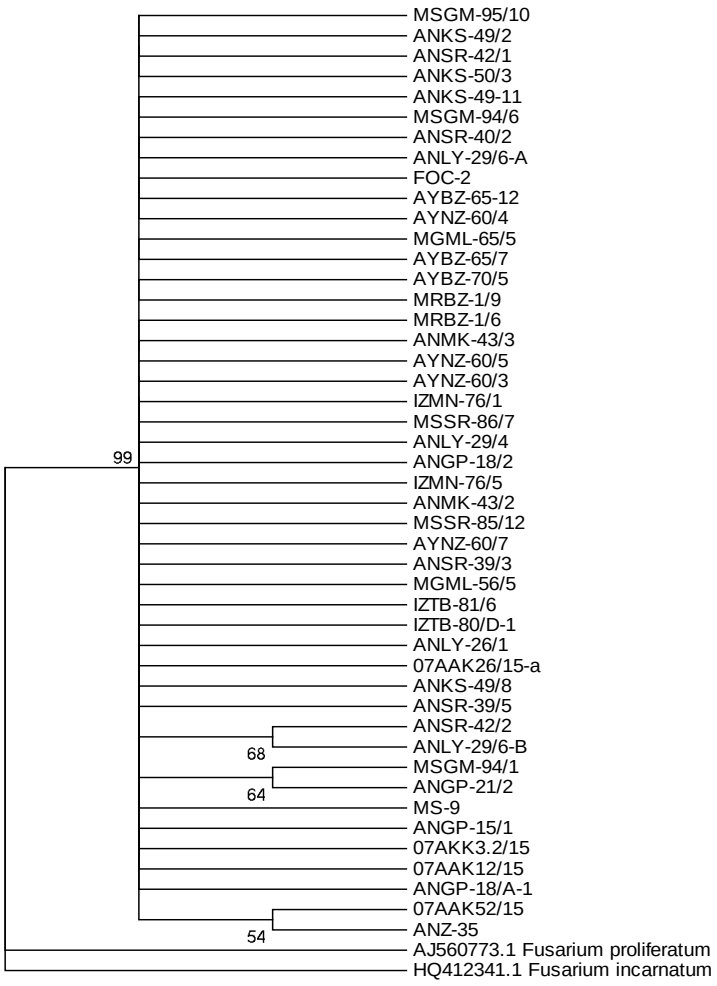

Figure 2. Phylogenetic tree constructed using the UPGMA clustering method with 2000 bootstrap replicates and drawn as condensed, based on sequences of the calmodulin (CAL) sequences gene regions of *Fusarium oxysporum* f. sp. *capsici* isolates (Foc). Non-host *F. oxysporum* Fol-07AAK52/15 and 07AKK3.2/15, Forl-07AAK26/15-a and 07AAK12/15, Fomg-MS-9 and ANZ-35. Out group Fusarium from GenBank *F. proliferatum*-AJ560773.1 and *F.incarnatum*-HQ412341.1.

CONCLUSIONS

The results obtained from the BT, CAL and CHS gene regions were not found to be informative for genetic diversity among Fomg and Foc sampled from pepper greenhouses/and fields in Turkey. On the other hand, sequence data for BT, CAL and CHS gene regions displayed limited variation among Fomg and Foc isolates and found inefficient to distinguish reliably among several FOSC. The sequence variation on these 'housekeeping gene' regions in the core genome was not considered adequate to differentiate FOSC populations. This study showed that the Fomg and Foc isolates exhibited possible monophyletic ontogeny, and there was no-significant variation in phylogenetic analyses of the BT, CAL and CHS regions to form genetically different groups. The uniform genetic structure of the Fomg and Foc populations in Turkey may be considered as an advantage in plant breeding studies to be carried out to generate resistant cultivars.

ACKNOWLEDGMENTS

This study is a part of the project fully supported by the Scientific and Technical Research Council of Turkey (TUBITAK-COST project number: 114O866).

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Original Scientific paper
10.7251/AGRENG1903100A
UDC 582.998.16:557.1(497.2)

CHEMICAL COMPOSITION OF CARDOON (*CYNARA CARDUNCULUS* L.) GROWN IN SOUTH BULGARIA

Violina ANGELOVA[1*], Mariana Perifanova NEMSKA[2], Galina UZUNOVA[2], Luzian KRUSTEV[2]

[1]Department of Chemistry, Agricultural University-Plovdiv, Bulgaria
[2]University of Food technology, Plovdiv, Bulgaria
*Corresponding author: vileriz@abv.bg

ABSTRACT

Comparative research has been conducted to allow us to determine the content of macro- and microelements in the vegetative and reproductive organs of *Cynara cardunculus* L. and quality of *Cynara cardunculus* L. oil. The experiment was performed on an agricultural field near Plovdiv (South Bulgaria). The contents of macro- and microelements in plant materials (roots, stems, leaves, seeds) and oils were determined. The oils were extracted using a Soxhlet apparatus from seeds of *Cynara cardunculus* L. The quantitative measurements were carried out with inductively-coupled plasma (ICP). Oil fatty acids characterization for unsaturated and saturated acids was performed by gas chromatography. The cardoon shows adaptability to local conditions and can be grown in southern Bulgaria and used for oil production. All plant parts of the cardoon are a rich source of macro and microelements and exhibit high nutritional value. The distribution of macro and microelements in the cardoon organs is selective, specific for the individual elements. Cd, Cu and Fe are accumulated in the roots, K - in the stems, Pb and Ca - in the leaves, and Zn, Mn, Mg and P - in the seeds. Cardoon seeds were a rich source of macro- and microelements (K, P, Mg, Ca, Fe, and Zn). Cardoon oil was abundant in unsaturated fatty acids (linoleic (61.67%%) and oleic acids (22.82%)), followed by palmitic acid (10.50%) and stearic acid (3.29%). Cardoon oil has a P/S index higher than 1 (4.2), which indicates that oil can have a good effect on human health and are oils suitable for consumption. Cardoon oil is a rich source of polyunsaturated linoleic fatty acid with potentially beneficial therapeutic activity.

Keywords: *Cardoon, fatty acid composition, micro and macroelements, oil, Bulgaria.*

INTRODUCTION

Cynara is a genus of thistle-like perennial plants of the Asteraceae family. They are found in the Mediterranean region, the Middle East, Northwest Africa and the Canary Islands. Among the known species of this genus are artichoke (Cynara Cardunculus Var. Scolymus (L.) Fiori) and cardoon, which is divided into

cultivated cardoon (C. Cardunculus Var. Altilis DC) and wild cardoon (C. Cardunculus Var. Sylvestris (Lamk) Fiori) (Foti et al., 1999; Portis et al., 2005; Ierna and Mauromicale, 2010).

C. cardunculus is a crop with various applications. Cardoon is rich in sugars, carotene, mineral salts, vitamins C, B1, B3, B5, B6, folic acid, Mg, Fe, Mn, Zn, and phosphorus. The edible parts of the plant are its unripe capitula (flowering heads), and fragile stalks and celery-flavoured leaf stalks that are usually consumed roasted, boiled, fried, or in salads (Fernández et al., 2006; Christaki et al., 2012). The aboveground biomass (leaves and stems) can be used as animal feed, for energy production (Mancini et al., 2019) and in the food industry (Almeida and Simões, 2018; Llorente et al., 2014). Cardoon leaves can be used to flavour alcoholic beverages (Foti et al., 1999) and its flowers for production of juices and high quality goat and sheep's milk cheese suitable for vegetarian consumers (Fernandez et al., 2006; Pino et al., 2009; Borgognone et al., 2014). Cardoon roots are a good source of inulin, which is used for nutritional and non-nutritional purposes (Ritsema and Smeekens, 2003). Cardoon seeds can be used for the production of biofuels (Gominho et al., 2011). Moreover, cardoon oil is suitable for human consumption because of its high nutritional value (Curt et al., 2002; Fernández et al., 2006; Raccuia и Melilli, 2007). Although cardoon is a non-wood plant, its stems can be used as a source of fibre (17% lignin) for paper production. Cardoon leaves are used for medical purposes because of their polyphenols content (Curt et al., 2002). Usually these leaves have a diuretic and hepatoprotective effect, they improve the function of the gallbladder, stimulate the secretion of digestive juices, especially bile, and can inhibit the cholesterol synthesis (Fernandez et al., 2006; Grammelis et al., 2008). Root extracts have significant antioxidant and antimicrobial properties that can be used for therapeutic and pharmaceutical purposes (Falleh et al., 2008). These physiological properties are due to the phenylpropanoids (flavonoids, mono- and dicaffeoylquinic acids) and sesquiterpene lactones (Lattanzio et al., 2009; Menin et al., 2010; Pandino et al., 2015) contained in them.

Cardoon, unlike artichoke, is a crop little known in Bulgaria. Cardoon is not grown in our country. Cardoon is a crop that is not demanding in terms of soil and can be grown without irrigation because it withstands drought. The climatic conditions in southern Bulgaria are suitable for its cultivation.

Most publications have focused on the evaluation of the use of cardoon seeds for biodiesel production, and there is insufficient information on the chemical composition of the cardoon.

The purpose of this research is to conduct a comparative study that will allow us to determine the quantities of macro and microelements in the vegetative and reproductive organs of the cardoon, the composition and quality of the oil, as well as to identify the possibilities for its cultivation in southern Bulgaria.

MATERIAL AND METHODS

The research has been carried out during the period 2017-2019. The study was conducted at the experimental field of Agricultural University-Plovdiv. The characteristics of soils are shown in Table 1. The soils are characterized by slightly alkaline reaction (pH 7.5) and average content of organic carbon (1.54%) and nutrients (N, P, K).

Table 1. Soil characteristics

pH	Organic carbon, %	Macroelements					Microelements and trace metals, mg/kg					
		N , %	K, %	Ca, %	Mg, %	P, mg/kg	Pb	Cd	Zn	Cu	Fe	Mn
7,5	1,54	0,13	0,68	1,6	1,0	354,9	24,6	2,7	33,9	16	27113	884,2

The test plant was cardoon (*Cynara cardunculus* L). Cardoon seeds were sown to a depth of 3-4 cm; between row and within row distances were 70 and 30 cm, respectively. The analyses were made in the second year of the growing of the plants. On reaching commercial ripeness the plants of cardoon were gathered. The oil from cardoon was derived under laboratory conditions through an extraction method with Socksle's apparatus, allowing the extraction of the oil from the ground seeds of cardoon by using petroleum ether and the subsequent liberation of the latter through distillation. Oil fatty acids characterization for unsaturated and saturated acids was performed by gas chromatography. The contents of trace metals, micro and macroelemens in different parts of cardoon (roots, stems, leaves, seeds), and oils were determined by the method of the microwave mineralization. Total content of trace metals in soils was determined in accordance with ISO 11466. The quantitative measurements were carried out with inductively coupled plasma emission spectrometry (ICP) (Jobin Yvon Emission - JY 38 S, France).

RESULTS AND DISCUSSION

Accumulation of trace metals, micro and macroelements in vegetative ad reproductive organs of cardoon

Table 2 shows the results obtained for the content of macro and microelements in the vegetative and reproductive organs of the cardoon. All plant parts of the cardoon are a rich source of macro and microelements and exhibit high nutritional value, as differences in the content of the studied elements are observed in the vegetative and reproductive organs of the plant. The distribution of trace metals, macro and microelements in the cardoon organs is selective, specific for the individual elements. Cd, Cu and Fe are accumulated in the roots, K - in the stems, Pb and Ca - in the leaves, and Zn, Mn, Mg and P - in the seeds (Fig. 1).

Table 2. Content of trace metals, micro and macroelements (mg/kg) in cardoon

	Roots	Stems	Leaves	Seeds	Oil
Pb	1,8	0,85	5,7	0,84	0,02
Cd	0,15	0,004	0,09	nd	nd
Zn	17,8	11,0	28,9	35,2	0,63
Cu	27,1	1,9	4,8	11,4	0,64
Fe	250,6	23,0	117,9	51,1	4.0
Mn	11,5	1,9	7,5	18,0	0,15
K	5127,8	14724,7	11596,9	6670,4	71,1
Ca	4118,2	3045,7	11676,3	2826,1	29,6
Mg	1765,2	255,7	3146,7	3572,3	10,9
P	847,3	1100,1	888,3	5398,2	12,7

nd-not detected

The content of Pb in cardoon stems and leaves varies from 0.85 to 5.6 mg/kg, Zn from 11.0 to 28.9 mg/kg, Cd from 0.003 to 0.09 mg/kg, Cu from 1.9 mg/kg to 4.8 mg/kg, Fe - 23.0 to 117.9 mg/kg, Mn - 1.9 to 7.5 mg/kg, K - 14724.7 to 11596.9 mg/kg, P - 1100 mg/kg to 888.3 mg/kg, Mg - 255.7 to 3146.7 mg/kg and Ca - from 3045.7 mg/kg 3146.7 mg/kg.

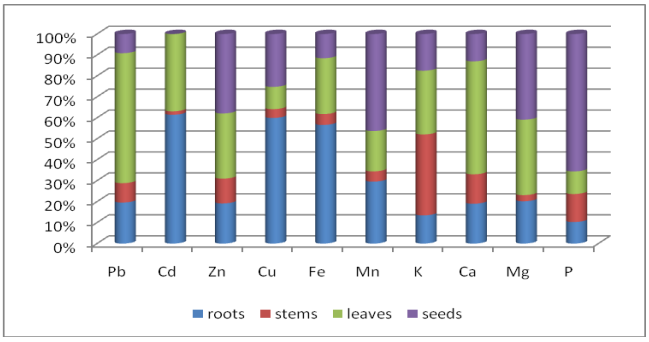

Fig. 1. Distribution of trace metals, micro and macroelements in cardoon

Significantly higher amounts of K (24000 mg/kg), Ca (26620 mg/kg), Mg (1910 mg/kg), Fe (230 mg/kg) and Mn (1910 mg/kg) were established by Petropoulos et al., 2018 in the cardoon leaves cultivated in southern Greece. Higher values for K (31700-34900 mg/kg), Mg (4500 mg/kg) and Ca (17000 mg/kg) in the leaves of hydroponically grown cardoon plants were also found by Rouphael et al. (2012) and Borgognone et al. (2014), while Colla et al (2013) report significant differences in the mineral composition of different genotypes sgrown under saline environment.

Variation between results may be due to cultivation conditions (hydroponic, greenhouse, and field trials), as well as with the differences in plant age, as the above studies refer to young plants and 5-year-old plants (Petropoulos et al., 2018).

Cardoon seeds can be a good source of minerals due to the high content of Ca, K, Mg and P in them. Macroelements (K, P, Mg, and Ca), followed by Fe and Zn, predominate in cardoon seeds. The content of Cu and Mn is significantly lower. The seeds also contain Pb, as its content in the seeds is significantly lower than that in the roots and the above-ground mass of the plants, while Cd is not accumulated in the seeds (below quantitative limits). Similar are the results of Petropoulos et al (2018), who found significant amounts of K, Ca, Mg and Fe in cardoon seeds, as Ca content reaches up to 11970 mg/kg, K up to 6630 mg/kg, Mg up to 4830 mg/kg, Fe up to 130 mg/kg, Zn up to 39.5 mg/kg, Na up to 180 mg/kg, and Mn up to 60 mg/kg. The values we obtained for K do not differ significantly from the results of Petropoulos et al. (2018). The content of Zn, Fe and Mg is slightly lower, while significantly higher values are obtained for Mn and Ca. The variation between the results may be due to growing conditions, genetic factors, varietal characteristics and other factors.

The content of P and Mg is highest in the cardoon seeds compared to other parts of the plant, while the content of K is lower than that in the stems and leaves. Similar results were established by Petropoulos et al., 2018, according to whom the content of K (6530 mg/kg) and Na (180 mg/kg) is lower than that in other parts of the plant, while higher levels of Mg (4830 mg/kg) were detected in the seeds.

Oil was obtained from the cardoon seeds in laboratory conditions by an extraction method with a Soxhlet apparatus, allowing the extraction of the fat from the pre-ground seeds with petroleum ether and subsequent distillation of the latter.

The content of Pb in cardoon oil reaches up to 0.02 mg/kg (Table 2). The maximum allowable concentration (MAC) for Pb in the vegetable oil is 0.1 mg/kg. The results obtained show that a very small amount of Pb contained in the cardoon seeds goes into the oil obtained, and its content in the oil is lower the MAC and it can be used for nutritional purposes.

The content of Cd is below the limits of the quantitative method used. According to the current standard, the content of Cd should not exceed 0.05 mg/kg.

The MAC for the content of Zn in vegetable fat is 10 mg/kg. The results obtained clearly show that the major part of Zn contained in the cardoon seeds does not do into the oil obtained, and its content in the oil is lower than the MAC.

The MAC for the content of Cu in refined oils is 0.1 mg/kg and in unrefined oil is 0.4 mg/kg. In our studies, the content of Cu in oil reaches 0.64 mg/kg and its quantity is above the MAC. It is noteworthy that although the content of Cu in the seeds is low, the oil contains Cu above the MAC. Probably the reason for this is the way the oil is extracted from the cardoon seeds. In terms of our experiment, the oil was obtained by extraction. However, there is evidence in the literature that shows that oils obtained by solvent extraction contain higher values of Cu and trace metals than cold-pressed oils. It is not desirable for the oil composition to contain significant amounts of microelements, in particular Cu. Cu ions are known to be effective pro-oxidants in the oxidation of lipids, so they are undesirable components in terms of oil resistance to oxidation. This is probably the reason for

the high criteria in terms of the content of Cu in oils, although Cu is not a toxic element to human health in a relatively wide range.

The content of Fe in cardoon oil reaches 4.0 mg/kg and is within the limit values for oils (for crude oils 5 mg/kg, for refined oils 1.5 mg/kg). The content of Fe in crude oils is due to the Fe contained in oilseeds (mainly related to proteins, phospholipids and other components).

Of the nutrients in the composition of cardoon oil, K (71.1 mg/kg) predominates followed by Ca (29.6 mg/kg), P (12.7 mg/kg) and Mg (10.9 mg/kg) (Table 4). There is no evidence of the negative impact of these elements on the stability of the oil. It is known that P and Ca form salts that are insoluble in oil and can be easily removed by refining the oil.

Fatty acid composition of cardoon oil

The fatty acid composition of the oil is the main factor that determines the use of the oil for nutritional purposes, for industrial purposes, as the variety, climate and production area have a significant impact (Velasco et al., 2005). Raccuia et al. (2011) found that cardoon oil is a rich source of unsaturated fatty acids such as linoleic and oleic acids (44.5 and 42.6%, respectively), while the content of saturated fatty acids such as palmitic and stearic acid is much lower (9.8 and 3.1%, respectively). Similar results were obtained by Petropoulos et al. (2018), according to whom linoleic and oleic acids (64.86 and 21.11%, respectively) predominate in cardoon oil, while palmitic and stearic acids are in smaller quantities (9.37 and 2.78%, respectively). Similar are the results of Maccarone et al. (1999) and Curt et al. (2002) according to whom linoleic and oleic acid are the main fatty acids, followed by palmitic and stearic acid. The composition of cardoon oil has been found to be similar to sunflower oil (Benjelloun-Mlayah et al., 1996; Curt et al., 2002; Fernández et al., 2006).

Table 3. Fatty acid composition of cardoon oil (expressed as % of total fatty acid composition)

Parameter	Measured	Reference				
		Greece(1)	Greece(2)	Italy(3)	Spain(4)	Portugal(5)
Saturated (S)	14.83	13.23				
Lauric acid (C12:0)	0.15	nd				
Myristic acid(C14:0)	0.05	0.094				
Palmitic acid (C16:0)	10.6	9.37	11.1	7.7-10.3	10.6	10.9
Magaric acid (C17:0)	0.08	0.072				
Stearic acd (C18:0)	3.3	2.72	3.2	2.8-3.6	3.56-3.7	3.3
Arachidic acid (C20:0)	0.15	0.277				
Lignoceric acid(C24:0)	0.5	0.121				
Monounsaturated(MUFA)	23.4	21.34				
Palmioletic acid(C16:1)	0.19	0.107				
Oleic acid (C18:1)	22.85	21.11	24.9	21.8-26.1	24.9-28.4	23.1
Gadoleic acid (C20:1)	0.36	0.11				
Polyunsaturated(PUFA)	61.77	65.43				
Linoleic acid(C 18:2)	61.69	64.86	59.1	61.2-	56.7 -	61.2

				62.7	59.7	
Linolenic acid (C 18:3)	0.08	0.108				
Total unsaturated (U)	85.17	86.77				
Saturated:unsaturated	14.83: 85.17	13.23: 86.77				
P/S index	4.2	4.94				

(1) Petropoulos et al. (2018); (2) Archontoulis et al. (2010); (3) Maccarone et al. (1999), Piscioneri et al. (2000), Raccuia and Melilli (2007); (4) Curt et al. (2002): (5) Carvalho et al. (2006)

Unsaturated fatty acids are predominant in the fatty acid composition of the oil we study, obtained during the extraction of cardoon seeds, with their amount reaching up to 85.06% respectively. The composition of the oil was dominated by linoleic acid (C18:2, 61.67%), followed by oleic acid (C18:1, 22.82%). The presence of linoleic (C18:3, 0.06%), palmitic (C16:1, 0.07%) and gadoleic (C20:1, 0.34%) acids was also found. Similar results are obtained by Petropoulos et al. (2018), according to whom the linolenic acid content is less than 1%. The low percentage of unsaturated acids with three double bonds, less than 1%, has a positive effect on the thermal and oxidative stability of the oil.

Of the saturated fatty acids, palmitic acid (C16:0) prevails in the amount of 10.5%, followed by stearic acid (C18:0, 3.29%). The oil also contains myristic (C14:0, 0.03%), margaric (C16:0, 0.06%), arachidonic (C20:0, 0.13%) and lignoceric (C24:0, 0.40%) acids. The content of saturated fatty acids in cardoon oil reaches 14.83% (Table 3).

The results obtained confirm that linoleic acid is predominant in cardoon oil (Maccarone et al.,1999, Piscioneri et al., 2000, Curt et al., 2002, Carvalho et al., 2006, Raccui and Melilli, 2007, Archontoulis et al., 2010, Petropoulos et al., 2018). According to the literature, the content of linoleic acid varies from 56.7% to 64.8%. Oils from Spain contain up to 59.7% linoleic acid (Curt et al., 2002), while oils from Italy, Portugal and Greece contain higher amounts of linoleic acid (61.2-64.8%) (Maccarone et al.,1999, Piscioneri et al., 2000, Carvalho et al., 2006, Raccui and Melilli, 2007, Archontoulis et al., 2010, Petropoulos et al., 2018)..

Oleic acid content was found to vary from 21.11% to 28.4% (Maccarone et al.,1999, Piscioneri et al., 2000, Curt et al., 2002, Carvalho et al., 2006, Raccui and Melilli, 2007, Archontoulis et al., 2010, Petropoulos et al., 2018). The content of palmitic acid in oil varies from 9.37% to 11.1%, while the content of stearic acid in oil varies from 2.78 to 3.7%.

The distribution of fatty acids is shown in Figure 2. The ratio of unsaturated to saturated fatty acids in cardoon oil is 85.17:14.83. Similar results were obtained by Petropoulos et al. (2018) for cardoon oils from southern Greece 86.77: 3.23.

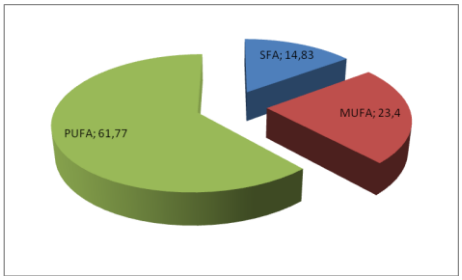

Fig.2**.** Distribution of fatty acids in cardoon oil

The total content of saturated fatty acids (SFA) in cardoon oil reaches 14.83% of the total amount of fatty acids and is comparable to the amounts of these acids in oils from Greece (Petropoulos et al., 2018). The content of monounsaturated fatty acids (MUFA) reaches up to 23.5% and the content of polyunsaturated fatty acids reaches up to 61.7%. The high PUFA content in cardoon oil makes it useful for therapeutic purposes in cardiovascular diseases. PUFAs are useful for reducing the risk of certain chronic conditions such as coronary heart disease, stroke and rheumatoid arthritis (Calder, 2008) and are used in the treatment of certain chronic conditions such as diabetes, cardiovascular diseases, inflammatory processes, atherosclerosis (Finley and Shahidi, 2001).

The relationship between the content of saturated and polyunsaturated acids is expressed as a P/S index. This value is an important parameter in determining the nutritional value of certain types of oils. Oils with a P/S index greater than 1 are considered valuable edible oils. The results obtained by Petropoulos et al. (2018) show that cardoon oil has a P/S index higher than 1 (4.94), which is in line with our results (4.2). These values indicate that cardoon oils can have a good effect on human health and are oils suitable for consumption.

CONCLUSIONS

Based on the obtained results the following conclusions can be made:

1. The cardoon shows adaptability to local conditions and can be grown in southern Bulgaria and used for oil production
2. All plant parts of the cardoon are a rich source of macro and microelements and exhibit high nutritional value, as differences in the content of the studied elements are observed in the vegetative and reproductive organs of the plant.
3. The distribution of macro and microelements in the cardoon organs is selective, specific for the individual elements. Cd, Cu and Fe are accumulated in the roots, K - in the stems, Pb and Ca - in the leaves, and Zn, Mn, Mg and P - in the seeds
4. Cardoon seeds can be a good source of minerals due to the high content of K(6670.4 mg/kg), P (5398.2 mg/kg), Mg (3572.3 mg/kg), Ca (2826.1 mg/kg), Fe (51.1 mg/kg), and Zn (35.2 mg/kg) in them.
5. Polyunsaturated fatty acids (PUFA-61.77%) are predominant in the fatty acid composition of the oil, followed by monounsaturated fatty acids (MUFA -23.4%) and saturated (SFA-14.83%).

6. Cardoon oil has a P/S index higher than 1 (4.2), which indicates that oil can have a good effect on human health and are oils suitable for consumption.
7. Cardoon oil is a rich source of polyunsaturated linoleic fatty acid with potentially beneficial therapeutic activity.

ACKNOWLEDGEMENT

The financial support by the Bulgarian National Science Fund Project DFNI H04/9 is greatly appreciated.

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Original Scientific paper
10.7251/AGRENG1903111M
UDC 546.23:582.929.4

EFFECT OF FOLIAR APPLICATION OF SELENIUM ON ITS UPTAKE AND YIELDS IN BASILS

Ivana MEZEYOVÁ*[1], Alžbeta HEGEDŰSOVÁ[1], Ondrej HEGEDŰS[2], Ján FARKAŠ[1], Miroslav ŠLOSÁR[1], Ján MEZEY[1]

[1]Slovak University of Agriculture in Nitra, Slovak republic
[2]Janos Selye University, Komárno, Slovak republic
*Corresponding author: ivana.mezeyova@uniag.sk

ABSTRACT

Basil is one of the most used spices in Slovakia. Selenium is an essential element for normal growth and development of the organism and because Slovak soils are poor in this element, the various ways of this antioxidant increasing in the food chain has being sought. The aim of the work was to evaluate influence of selenium biofortification on selenium content of *Ocimum basilicum* - variety ´Dark Green´, which was, in conditions of the Slovakia, well known and wide spread grown, as well as on the opal basils (´Purple Ruffles´ and ´Red Rubin´) and on *Ocimum tenuiflorum* – Tulsi. The influence of fortification on the yields of basils was also tested. The selenium content and the yields of selected basils were compared in dependence on the selenium fertilization, two terms of harvest and morphological variability. Small-scale field experiment was carried out at the Department of Vegetable Production, Slovak University of Agriculture in Nitra, 2016. Selenium was applied foliar at a dose 50 g Se / ha in the form of sodium selenate. In two harvests the values of selenium built in plants and the economically interesting quantitative data – the yields per ha were evaluated. Statistical methods were used for statistical evaluation by the help of Statgraphics Centurion XVII (StatPoint Inc. USA), with multifactor analysis of variance (ANOVA) and LSD test. Foliar application of selenium had a positive effect of selenium content in case of all tested basils. The yields were also positively affected where values of fresh mass in selenised variants ranged from 1.43 (´Purple Ruffles´) to 13.71 t/ha (Tulsi).

Keywords: *basil, selenium, yields, fortification, fertilization.*

INTRODUCTION

Basil (*Ocimum spp.*) is a common herb that is known for its ornamental and therapeutic importance. Basil has been grown traditionally in worldwide as a decorative, medicinal, seasoning and ritual herbs (Cherian, 2019). The chemical constituents which have been isolated from the plant include terpenoids, alkaloids, flavonoids, tannins, saponin glycosides and ascorbic acid. It has been reported to be hepatoprotective, immunomodulatory, antihyperglycemic, hypolipidemic,

antitoxic, anti-inflammatory, antibacterial and antifungal (Khair-ul-Bariyah et al., 2012). Sweet basil (*Ocimum basilicum* L.) and holy basil (*Ocimum sanctum* L.) are the most widely grown basil species in the world either for the fresh market or for essential oil production (Zheljazkov et al., 2008). According to the current botanical database The Plant list, 2019 there are 66 accepted basil species within the family *Lamiaceae*. The different species and varieties of this family have different yields properties. Selenium is biologically active at low concentrations for normal growth and development, and at moderate concentrations for homeostatic function (Hamilton, 2004). In human, low dietary selenium intakes are associated with health disorders including oxidative stress-related conditions, reduced fertility andimmune functions and an increased risk of cancers (Gebreeyessus and Zewge, 2019). Recommended appropriate dietary Se intake for healthy adult men is 80 μg day-1 and 55 μg day-1 for adult women, whereas a maximal daily safe dietary Se intake up to 400μg has been suggested for adults by WHO/IPCS (Ozkutlu et al., 2011). Biofortification of edible crops with selenium (Se) may represent an alternative system for providing selenium in the human diet. Currently there is growing interest in Se and its effects on plant metabolism, biofortification, phytoremediation, and plant tolerance to stress conditions (Oraghi Ardebili et al., 2015). Studying the dynamics of Se plant uptake is crucial in controlling the Se content in plants and in reducing the risk of both Se toxicity and deficiency. The addition of selenate to the nutrient solution could be an efficient system for providing enriched basil plants (Puccinelli et al., 2017).

The aim of the work was to evaluate the influence of selenium biofortification on the content increasing of selenium in plants as well as its impact on important quantitative data – the yields per ha.

MATERIALS AND METHODS

Field trial

Three varieties of *Ocimum basilicum* - 'Red Rubin', 'Purple Ruffles' and 'Dark Green' were included in the small-scale field trial, as well as the basil Tulsi (*Ocimum tenuiflorum*). Sowing was carried out in the greenhouses of the Slovak University of Agriculture demonstration garden on March 8, 2016. Planting on a permanent site was carried out on May 16, 2016, when the risk of spring frosts was reduced to a minimum. In each variant, 10 plants were planted in 3 reps in spacing of 0.35 x 0.35 m. The basil growth was regularly treated with soil removal and loosening. Based on the soil analysis (Table 1) into the soil during the vegetation, the ammonium liqueur (LAD 27) was applied in an amount of 0.4 kg in two doses. The first dose was applied about two weeks after planting; the second dose was applied directly to the plants after the first harvest on July 12, 2016. On June 3, 2016 Actara spray was applied in the amount of 0.40 g to 2 litres, as there were flea beetles on the basil plants. The herbs were harvested two times during vegetation in the phase of flowering start (BBCH 61) on June 12, 2016 and August 26, 2016. Harvesting was done by hand with scissors, in the morning with no wind and sunny

weather. Immediately the herbs were weight and counted to tone per hectare, and the mas was dried in hall by air for the selenium estimation.

Table 1 Results of soil compounds in trial area, mg.kg-1, Nitra, 2016

pH	Nan mg.kg-1	Nutrients content in mg.kg^{-1} (Mehl.III)				S	% humus
		P	K	Ca	Mg		
7,17L	13,0S	142,5L	565VH	14750VH	740,9VH	16,3L	4,14H

Explanatory Notes: pH: N – neutral, nutrients: VL – very low content, L – low content, H – high content, VH – very high content

Table 2 Evaluation of average monthly precipitation (P) and temperatures (t) in 2016 according to long-term climatic norm 1961-1990, Nitra

Month	P [mm]	**characteristic**	t [ºC]	**characteristic**
V.	91	very moist	15.0	normal
VI.	14	extremely dry	20.3	very hot
VII.	135	extremely moist	21.4	hot
VIII.	35	dry	19.5	normal

Biofortification with selenium

Six weeks after planting in BBCH 61 stage – beginning of the flowering, sodium selenate at a dose of 50 g Se / ha was applied to the leaves of the plants in a light sunny weather in two variants (control and applied selenium).

Selenium content estimation

The total content of selenium was determined in a digestion of plant material patterns. Quantitative determination of selenium was done by using of ET-AAS method with Zeeman background correction. Atomic absorption spectrometer SpectrAA240FS (Varian, Mulgrave Virginia, Australia) was used to measure the total selenium content. Conditions for selenium measurement were set in the equipment according to the recommendations of the manufacturer (Rothery, 1988) for ET-AAS technique.

Yields estimation

After each harvest of basil, the weighing of each variant was carried out in laboratory of Department of Vegetable Production, SUA, Nitra. The average weight of the fresh mass was weighed in grams and then re-counted in t.ha^{-1}.

Statistical analyses

The analysis of variance (ANOVA), the multifactor analysis of variance (MANOVA) and the multiple Range test were done using the Statgraphic Centurion XVII (StatPoint Inc. USA).

RESULTS AND DISCUSSION

Selenium content

Selenium biofortification with foliar-applied sodium selenate solution significantly increased ($P > 0.05$) the content of selenium in plants in first harvest (table 3). The increasing was recorded from 0.189 to 6.392 mg/kg DM in average, what are about 33.8 - fold higher values in comparison to control variant. The highly evident difference in the first harvest data confirms the theory that selenium from sodium selenate application is incorporated during the first days after application. Foliar application is useful in that selenium does not enter the soil as it would be detected in biomass during the second harvest. In this way, there is no contamination of the soil, but its incorporation into the plant, which is confirmed by the significant differences between the variants in the first harvest and the fact that the selenium increase was not detected in the second harvest. The difference between first and second harvest was significant ($P > 0.05$) according to used statistical analyses (table 3), since the fortification did not take place after first harvest. Though, selenium fortification is important for normal human physiology in selenium deficient environments, it needs to be minimized or removed from selenium rich environmental media (Gebreeyessus and Zewge, 2019). Results indicated that the 50g/ha concentration of sodium selenate application in the form of foliar spray significantly enhanced the selenium content in garlic bulb (3.23±0.16mgSe/Kg) and vegetative part (15.46±0.71mgSe/Kg) where a 12.52 and 7.8 fold increase was observed respectively, as compared to control by Shafiq et al. (2019). According to Kopsell et al. (2009) selenization was effective for basil and cilantro grown in both a controlled environment and a field environment. Tissue Se concentrations in basil and cilantro increased in response to increasing foliar Se treatment concentrations from both selenate-Se and selenite-Se forms in both environments. Maximum Se tissue accumulation across both Se forms for basil and cilantro in the controlled environment averaged 55.0 and 33.9 $\mu g \cdot g^{-1}$ Se DM, respectively.

The varietal variability also had significant influence on building of selenium in observed plants (Figure 1). The variety ´Purple Ruffles´ built 14.38 mg/kg DM of selenium in to its tissues in first harvest. When comparing to average data of all observed basil, this difference was significant ($P > 0.05$) when comparing to Tulsi and ´Dark Green´. According to Ozkutlu et al. (2011) from the analysed 26 medicinal and aromatic plants, the highest Se concentration (1133 $\mu g\ kg^{-1}$) was found in sweet basil (*Ocimum basilicum* L.) and the lowest in sumac (*Rhus coriaria* L.) fruits (11 $\mu g\ kg^{-1}$). In previous studies, Se contents of some plants are reported as follows; Brazil nuts (14,700 $\mu g\ kg^{-1}$) and mosses growing in the Scandinavian countries (390 $\mu g\ kg^{-1}$- 2900 $\mu g\ kg^{-1}$).

Table 3: Effect of selenium biofortification and harvest on selenium content (mg/kg) in dry matter (DM) of selected basil, Nitra, 2016

		Variant	´Purple Ruffles´	´Red Rubin´	Tulsi	´Dark Green´	Average
1.	**harvest**^{A}	control	0.22±0,14	0.16±0.1	0.26±0.12	0.11±0.0	0.189±0.065^{a}
		Se	14.38±2.1	5.12±0.8	3.72±0.4	2.34±0.6	6.392±5.446^{b}
2.	**harvest**^{B}	control	ND	0.03±0.02	0.10±0.09	ND*	0.032±0.046^{a}
		Se	0.04±0.02	0.8±0.41	0.08±0.01	ND*	0.051±0.038^{a}

A, a - Column values with different lowercase letters in superscript are significantly different at P < 0.05 by LSD test in ANOVA (Statgraphic XVII)
**not detectable*

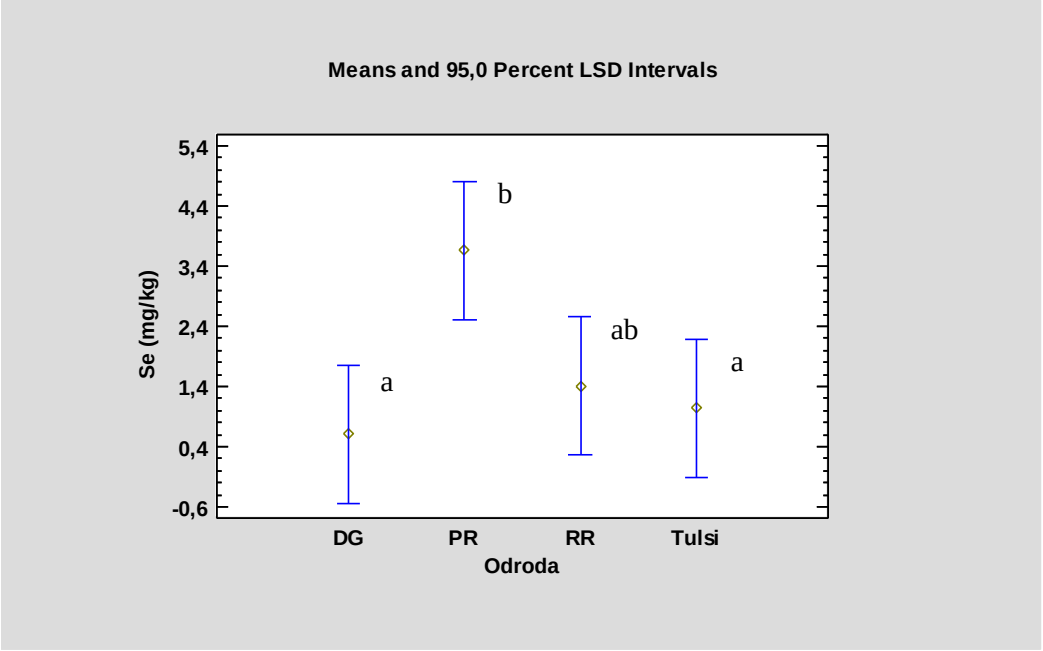

Figure 1 Effect of varietal variability on selenium content in dry matter (DM) of selected basil, Nitra, 2016

Yields

The yields of the basils moved from 1.05 t/ha (´Purple Ruffles´ in first harvest, control variant) to 13.71 t/ha (Tulsi, in second harvest, selenised variant) according to table 4. In comparison of average data the values of the yield in fortificated variant were higher in comparison to control, but this increasing wasn´t significant according to used statistical analyses (P < 0.05). El-Ramady et al. (2015) states that while there is no evidence of Se need for higher plants, several reports show that when Se added at low concentrations, Se exerts beneficial effects on plant growth.

Se may act as quasi-essential micronutrient through altering different physiological and biochemical traits. Thus, plants vary considerably in their physiological and biochemical response to Se. The concentration we tested did not inhibit or weaken the plants in their health condition. Oraghi Ardebili et al. (2015) observed the effect of increasing selenium dose (0 $mg.L^{-1}$; 30 $mg.L^{-1}$; 60 $mg.L^{-1}$; 120 $mg.L^{-1}$) on weight (g) of fresh and dry basil leaves. In their research, they found that leaf weight decreased in the case of increasing selenium doses. A dose of 120 mg. L^{-1} caused leaf growth inhibition and caused necrotic lesions on the leaves. The basil exhibited signs of toxicity at such high selenium concentration. In selenium biofortification, it is very important to define the dose very well, as it may be toxic when it is increased. According to Hawrylak-Nowak (2008), the foliar application of selenium in the form of sodium selenite can be an effective way of enriching the phytomass of basil with this element. Selenium in a wide range of concentrations (1-50 mg Se/dm^3) did not cause plant damage and only slightly affected the analysed physiological parameters. The results showed that Se addition mitigated the detrimental effect of salinity on lettuce growth and its development. Se application (100 ppm) increased also the head weight, leaf area, leaves dry weight and chlorophyll content by 46.4, 66.4, 61.8 and 31.5%, respectively compared to control plants in study of Shalaby et al., 2017. According to Khalid et al. (2017) it may be concluded that treated chives varieties with Se doses improved the vegetative growth characters (VGC) and essential oils (EO) while photosynthetic pigments (PHP) {chlorophyll (Chl *a*, Chl *b* and total carotenoids (TC)} and major constituents of EO were changed. In nonaccumulator plants, stunting, necrotic lesions on the leaves and decreased root growth have been mentioned as symptoms of Se toxicity (Matich et al., 2009).

According to table 4 the highest values of the yield were found in second harvest, indicating that the harvest date has a significant influence ($P < 0.05$) on basil yield. This fact is characteristic for basil plants, because they are the long day plants and requires full sunshine and warmth during its vegetation. Sharma et al. (1987) states that basil has high demands for sunlight and daylight luminance for about 15 hours when it reaches the highest yield. The increasing in case of observed plants was in average (table 4) in control variant from 4.29 to 9.36 t/ha (about 118 %) and in selenised variant from 5.41 to 10.43 t/ha (about 93 %).

According to figure 2 the influence of variety was significant ($P < 0.05$) in case of ´Purple Ruffles´in comparison to other varieties. According to The plant list, 2018 is for genus basil typical very various variability, which is project also in to different yields. According to Majkowska-Gadomska et al., 2017 cinnamon and Greek 'Minette' basils were characterised by the highest fresh herbage yields. The yields of Thai and sweet basils were lower by 19.3 and 26.7%, respectively. Purple and lemon basils had the lowest fresh herbage yields.

Table 4. Impact of selenium biofortification and harvest on fresh matter (FM) of selected basil, Nitra, 2016

		Variant	´Purple Ruffles´	´Red Rubin´	Tulsi	´Dark Green´	average
1.	harvest^{A}	control	1.05±0.16	6.3±0.57	5.33±1.00	4.48±0.33	4.29±2.28^{a}
		Se	1.43±0.29	7.52±1.62	5.81±0.60	6.86±0.86	5.41±2.74^{a}
2.	harvest^{B}	control	3.81±0.59	11.24±2.88	11.9±4.79	10.48±1.08	9.36±3.74^{b}
		Se	4.00±1.25	12.57±1.31	13.71±2.49	11.43±3.87	10.43±4.38^{b}

A, a - Column values with different lowercase letters in superscript are significantly different at P < 0.05 by LSD test in ANOVA (Statgraphic XVII)

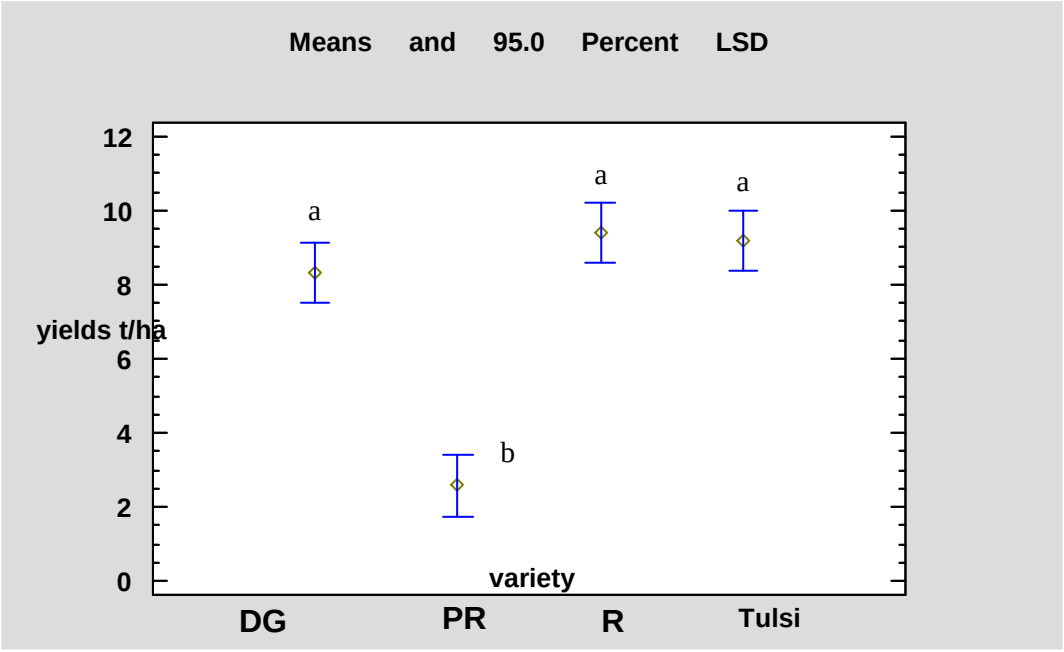

Figure 2 Effect of varietal variability on fresh matter crop (CH) of selected basil, Nitra, 2016

CONCLUSIONS

Antioxidants include selenium - an essential element needed for optimal growth and development of living organisms. It is not enough in Slovak soils and so possibilities of its increase in the food chain are sought. One of them is biofortification, respectively fertilization in the form of inorganic selenium, when it is in the case of a suitable crop metabolized into an organic form acceptable to humans. Several researches have confirmed that basil is very suitable for this purpose, as confirmed by the results of our research in the case of 'Dark Green', 'Purple Ruffles', 'Red Rubin' and *Ocimum tenuiflorum* - Tulsi. However, selenium

toxicity at high doses is one of the problems of such fertilization, which may affect the yields of selenised plants. Therefore, the appropriate dose and the ways of application should be selected. Dose of 50 g Se / ha did not inhibit the yields of selenised basils, the values were slightly higher in comparison to control variant.

ACKNOWLEDGEMENTS

The work was supported by project VEGA 1/0087/17, KEGA 012UKF-4/2019, AgroBioTech Research Centre built in accordance with the project Building „AgroBioTech" Research Centre ITMS 26220220180.

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Original Scientific paper
10.7251/AGRENG1903120Y
UDC 664.8:579.67

STUDY OF THE SURVIVAL OF *BACILLUS CEREUS* IN LOW-ACID CANNED VEGETABLES

Zinaida YEGOROVA*, Anastasia NIKITENKO

Belarusian State Technological University, Minsk, Belarus
*Corresponding author: egorovaze@tut.by

ABSTRACT

The species *B. cereus* is widely distributed in food products and is often the cause of food poisoning. Therefore, data on the ability of this microorganism to develop in low-acid canned products are of scientific and practical interest. The aim of the study was to determine the survival of *B. cereus* in low-acid canned vegetables stored at different temperatures. The objects of the study were industrial samples of aseptic canned carrot puree (pH = 5.11, a_w = 0.912) and sliced beets sterilized in vacuumized polymer bags (pH = 5.43, aw = 0.887). The *B. cereus* 11778 strain was used. The listed products were contaminated daily culture of the test-strain, with a concentration of 10^4–10^5 CFU/ml, and incubated at 6 and 30°C for 26 days. Sampling of products was carried out every 3 days to identify viable microorganisms. The generally accepted methods of experimental microbiology were used. It was found that in the canned "Beet sliced" *B. cereus* died after 10 days of storing the product at a temperature of 30°C. At a storage temperature of the contaminated product equal to 6°C, the test-microorganism did not die during the whole experiment and was detected in the amount of tens CFU in 1 ml of the product. In aseptic canned carrot puree, regardless of the storage temperature, *B. cereus* remained viable throughout the experiment, i. e. 26 days. The approximation of the obtained numerical values showed acceptable convergence of the curves with experimental data (R^2 = 0.9186 ÷ 0.985). Microscopic examination of Gram-stained *B. cereus* 11778 preparations isolated from contaminated samples of canned products during the experiment showed abundant sporulation of the test-strain. The research results can be used to predict the activity of *B. cereus* in canned vegetable products under various environmental conditions.

Keywords: *Canned vegetables, Bacillus cereus, Survival curves, Environmental conditions*

INTRODUCTION

Among the pathogens that are traditionally found in food, *Bacillus cereus*, aerobic, spore-forming gram and catalase-positive bacteria (Bergey, 1994), are able to survive in sterilized canned food. They are associated with food poisoning in Europe, at least since 1906 (Jay, 1996). The *B. cereus* group includes seven closely related species: *B. cereus sensustricto* (here called *B. cereus*), *B. anthracis*, *B.*

thuringiensis, *B. mycoides*, *B. pseudomycoides*, *B. weihenstephanensis* and *B. cytotoxicus* (Guinebretière *et al*., 2013). Numerous studies have shown that a large variability of properties is characteristic of this microorganism, therefore, according to aberrant and deviant strains are often encountered (Goeffert, Spira, Kim, 1972). The following data on the prevalence of *B. cereus* are given in studies describing studies of the contamination of food products with this microorganism.

Kjellander and Nygren (1957) examined 514 food samples and found that 26% of meat samples, 77% of milk samples, and 51% of fruits, nuts, and vegetables contained *B. cereus*. Andersson and Storgards (1959) reported that 129 of 486 samples of pasteurized milk, 147 of 161 samples of cream and 157 of 161 samples of whipped cream contained viable *B. cereus* cells. Nygren (1962) examined 3888 food samples and found that *B. cereus* contained in 51.6% of 1546 samples of food ingredients, in 43.8% of 1911 samples of cream and pudding, and in 52.2% of 431 samples of meat and vegetable products. In most cases, the level of contamination was less than 1×10^2 CFU/g (Goeffert *et al*., 1972). A study of selected dry products purchased at retail outlets in Madison, Wisconsin, revealed *B. cereus* in 25.3% of the products. Most often, this type of bacteria was found in spices, flavoring, dried potatoes, powdered milk and spaghetti sauces (Blackburn, 2008). So, studies of 20 species of spices showed that 71% of samples were contaminated with *B. cereus* (Merchina, 2003). There is evidence of the presence of *B. cereus* in canned food, sausage and confectionery (Vasiliev *et al*., 2013). It is believed that the optimum temperature for the development of this microorganism is 30–32°C, the maximum is 37–48°C, and the minimum is 10°C. However, in the literature there is evidence of the ability of *B. cereus* to multiply at 49°C and grow at 50°C. Studies performed by Halvorson *et al*. and Knaysi showed a difference in temperature limits during spore germination and vegetative growth of *B. cereus*. So, the temperature limits for spore germination were minus 1ºC (minimum), 30ºC (optimal) and 59ºC (maximum). It should be noted that this range is established in nutrient media containing mineral acids and alkalis as pH regulators (Jay, 1996). The type of acid affects the vital activity of *B. cereus*. Acetic acid has the greatest bacteriostatic effect. Growth delay of *B. cereus* with this acid is observed at pH = 4.5 and even 6.0. Acidification of products with other acids retards growth only at pH = 4.0 (Lindsay *et al*., 2000). A microbe can develop at a concentration of table salt in an environment of up to 10–15%, sugar up to 30–60% (Blackburn, 2008).

Of great practical interest is the thermal stability of *B. cereus* spores (Burgos *et al*., 1972, Ordonez and Burgos, 1976). Ingram (1969), referring to data from various sources, determined the value of D_{100} in food products with low acidity (pH> 4.5), equal to 5 minutes. There were published D values at 85ºC, 90ºC, 95ºC and 100ºC in phosphate buffer (pH = 7.0), namely: 220, 71, 13 and 8 min, respectively, the values of D_{121} in soybean and olive oil were 30 and 17.5 min, accordingly (Guinebretière *et al*., 2013). Concluding the brief review, it can be noted that, despite a certain scientific and practical groundwork in the field of predicting the activity of the *B. cereus* in various foods, there are still insufficiently studied questions of its survival in low acid canned foods from vegetables. Thus, the

objective of this work was to determine the survival of *B. cereus* in low-acid canned vegetables stored at different temperatures.

MATERIAL AND METHODS

The objects of study were food products (Table 1) and *Bacillus cereus* 11778 strain, kindly provided to us by the Department of Microbiology of the RSPC of Hygiene (Minsk, Belarus).

Table 1. Summary description of food products.

Food	Composition	Indicators				
		pH	Mass fraction,%:		a_w	Eh, mV
			soluble solids	titrated acids		
Sliced beets sterilized in vacuumized polymer bags	Beet roots, prepared water, citric acid	5.43	12.3	0.125	0.887	77.75
Aseptic canned carrot puree	Carrot roots, citric acid	5.11	7.47	0.07	0.912	95.2

In the experiments, daily culture *B. cereus* 11778 was used. For this purpose, for 18...20 h before testing, the test strain was sown on sloped nutrient agar. A suspension of the microorganism culture grown overnight was prepared on the day of testing. Determination of the survival of *B. cereus* in different food environments was carried out according to the plan given in Table 2. The results of microbiological studies were processed using methods of mathematical statistics (Garnayev, 1999, Alekseev *et al*., 2008). Additionally, we studied the change in the morphological properties of isolates of *B. cereus* during the experiment. For this purpose, the isolated cultures of the test microorganism were Gram-stained and microscopy.

Table 2. Experiment plan for determining the survival curves of *B. cereus* in different food environments.

Food and storage temperature, ºC*	The initial titer in product, CFU/cm^3	Sampling frequency	Thermostating conditions
Sliced beets sterilized in vacuumized polymer bags, 6 30	 1.1×10^5 1.1×10^5	Every 3 days during the 26 days	Nutrient agar, 30ºC, 48 h
Aseptic canned carrot puree 6 30	 8.6×10^4 1.7×10^4		

* Control (noncontaminated) food samples were stored in parallel.

Organoleptic (color, texture, odor) and physical and chemical (pH, mass fractions of soluble solids and titrated acids, water activity and redox potential) characteristics of control and experimental food samples were investigated at the end of the experiment. The pH value was measured with an accuracy of ± 0.01 using a pH-meter Hanna Instruments HI 2211-02 (GOST 26188). Mass fraction of titrated acids was determined by a potentiometric method using a pH-meter "Hanna Instruments HI 2211-02" and a glass electrode with a spherical membrane and a ceramic diaphragm with an accuracy of ± 1% (GOST ISO 750). Mass fraction of soluble solids was measured with an accuracy of ± 0.1% (GOST ISO 2173) using the refractometric method (Atago NAR-1T refractometer). The determination of water activity was carried out using a Roremeter RM-10 type water activity analyzer, the measurement accuracy was ± 0.2 (GOST ISO 21807). To determine the redox potential, the following measuring systems were used: an ionomer I–160 M, a platinum high-temperature electrode EVP-1 and a silver chloride reference electrode EVL-1M3.1 (accuracy class (error Δ (Eh) = ± 1mV); pH meter pH 210 manufactured by HANNA Instruments with a HI 3131 P combined electrode (accuracy class (error Δ (Eh) = ± 3mV).

RESULTS AND DISCUSSION

The results of studies of the survival of *B. cereus* in canned vegetables thermostatted at different temperatures are shown in Figures 1 and 2. As can be seen from the data (Figure 1), the death of the test-microorganism occurred after 10 days into canned sliced beet which was thermostated at 30°C, while at a temperature of 6°C, this test-microorganism was detected in the amount of tens of CFU in contaminated product after 26 days of storage. Other results were obtained in an experiment with samples of carrot mashed aseptic canning (Figure 2). Regardless of the storage temperature of samples of contaminated products, *B. cereus* did not lose viability throughout the experiment. However, unlike storage in canned sliced beets, the lowered temperature (6°C) had an inhibitory effect on the growth of the test-microorganism. So at the end of the experiment, the content of *B. cereus* in a contaminated carrot puree stored at 6°C did not exceed 10 CFU. In contaminated samples of carrot puree stored at 30°C for 26 days, the content of *B. cereus* increased by 3 orders of magnitude compared with the original titer.

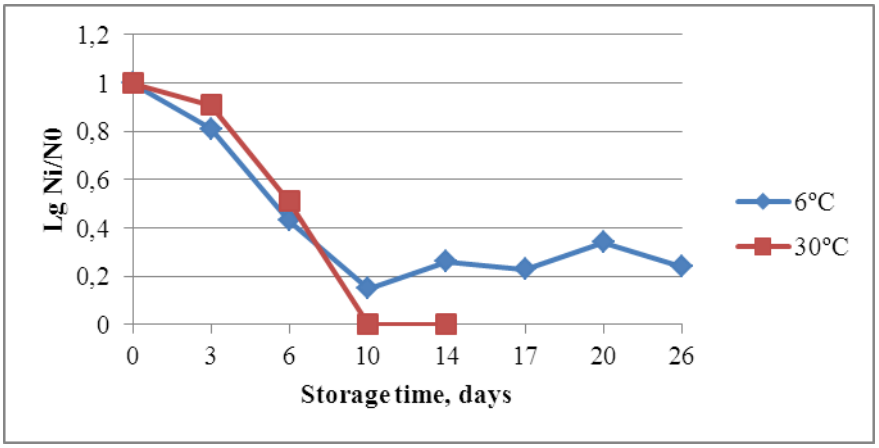

Figure 1. The survival of *B. cereus* 11778 into canned sliced beets, stored at different temperatures

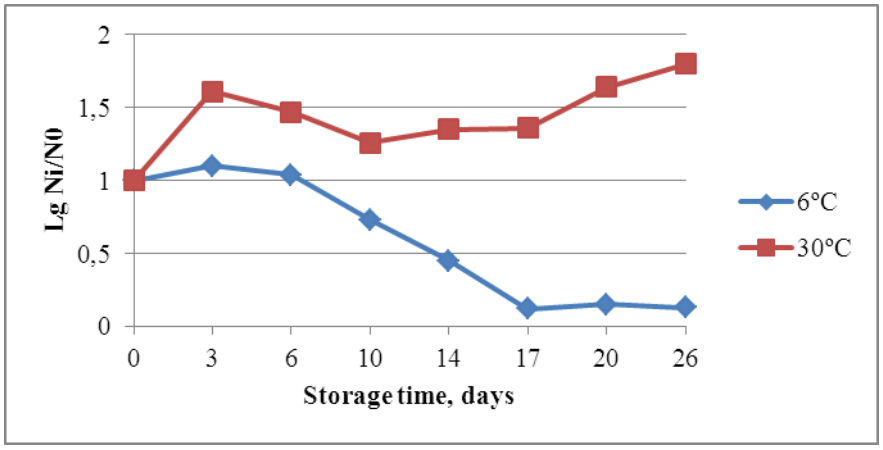

Figure 2. The survival of *B. cereus* 11778 into aseptic canned carrot puree, stored at different temperatures

The approximation of the experimental data on the change in the number of the test-strain we studied in two types of canned products stored at different temperatures allowed us to obtain equations describing the survival curves with different degrees of accuracy (Table 3).

Table 3. Prediction equations for *B. cereus* in low-acid canned vegetables thermostatted at different temperatures

Food	Temperature conditions, °C	Prediction equation	R^2 value
Sliced beets sterilized	6	$y = -1E\text{-}04x^4 + 0.0041\ x^3 - 0.0313x^2 - 0.4117x + 5.1565$	0.9695
	30	$y = -0.4162x + 5.1866$	0.9186
Carrot puree	6	$y = 0.0014x^3 - 0.0535x^2 + 0.2653x + 5.0146$	0.985
	30	$y = -1E\text{-}05x^5 - 0.001x^4 + 0.0295x^3 - 0.36x^2 + 1.6533x + 4.2586$	0.969

The results of changes in the physical and chemical parameters of the control and experimental samples of food products are given in Table 4 and indicate the following. The presence of *B. cereus* in canned sliced beets, stored in at temperature 6°C, caused a decrease in pH (1.2 times) and an increase in titrated acidity (one and a half times) and redox potential (almost 2 times). Analysis of the data obtained in the study of the physical and chemical parameters of the control and experimental samples of carrot puree showed that the greatest changes occurred in the value of the redox potential (decreased by 1.12–1.16 times) and water activity (decreased by approximately 0.03). At the same time, the consistency, color and smell of experimental samples of canned sliced beets and canned carrot puree remained unchanged.

Table 4. Physical and chemical parameters of the control and experimental samples of food products after 26 days of storage at different temperatures

Food	Kind	Temperature conditions, °C	Indicators				
			pH	Mass fraction,%:		a_w	Eh, mV
				soluble solids	titrated acids		
Canned sliced beets*	Control	6	5.49	11.54	0.22	0.9457	74.2
	Experimental	6	4.55	11.28	0.34	0.9457	127.3
Aseptic canned carrot puree	Control	6	5.2	7.09	0.106	0.973	90.2
		30	5.1	7.43	0.101	0.959	97.9
	Experimental	6	5.38	7.18	0.0744	0.945	80.4
		30	5.34	7.60	0.113	0.933	84.3

* Physical and chemical indicators in the control and experimental samples of sliced beets, stored at a temperature of 30°C, were not determined due to the premature termination of the experiment.

CONCLUSIONS

Based on the analysis of the sources of literature and the results of own experimental studies, we can create the following conclusions. At a temperature that is optimal for the development of *Bacillus cereus* (30ºC), its survival in low-acid canned vegetables (canned sliced beets and aseptic canned carrot puree) depended on the composition of the nutrient medium. The temperature of thermostating equal to 6 ° C led to the death of *Bacillus cereus* 11778 in canned sliced beets after 10 days of storage and gradual dying off in aseptic canned carrot puree after 26 days of storage (the number of test-microorganism did not exceed a few CFU/g).

The obtained data on the survival of Bacillus cereus 11778 into canned sliced beets and aseptic canned carrot puree can be used to risk assessment of their growth at different stages of the production process and use, as well as to develop appropriate control measures.

ACKNOWLEDGEMENTS

This study was carried out within the framework of the State pro-gram of scientific research "Biotechnology" for 2016-2020, subroutine "Microbial biotechnology“ and applied works for some of the Belarusian canneries.

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Original Scientific paper
10.7251/AGRENG1903128F
UDC 631:316.334.55(669)

EFFECTIVENESS OF COMMERCIAL AGRICULTURAL DEVELOPMENT (YCAD) PROGRAMME AMONG RURAL YOUTH IN EKITI STATE, NIGERIA

Olajide Julius FILUSI, Julius Olatunde AYINDE*

Department of Agricultural Extension and Rural Development, Faculty of Agriculture, Obafemi Awolowo University, Osun State, Ile-Ife, Nigeria
*Corresponding author: tundeyjoy@yahoo.com

ABSTRACT

The study described the socio-economic characteristics of the beneficiaries of the Youth Commercial Agricultural Development (YCAD) Programme in Ekiti State, Nigeria.Specifically, it identified type of enterprises in YCAD programme and isolated the factors influencing the effectiveness of the programme in the state. Multistage sampling procedure was used to select 174 beneficiaries/respondents for the study. A validated interview schedule was used to collect data which were summarised with percentages, means and standard deviation while chi-square and correlation were used to draw inferences. Also, factor analysis was used to isolate factors influencing the programme effectiveness. Results showed that the mean age of respondents was 37±5years, mean household size was 5±2 persons, mean year of formal education was 15±2 years and mean monthly income was ₦41,000±23,000. Results, also, showed that arable crop enterprises (47.7%) and poultry (27%) were the most preferred enterprises by the beneficiaries in the study area followed by tree crops enterprise (12.6%) and aquaculture (12.6%)respectively. In addition, five crucial factors such as Institutional factor (26.672%), Personnel factor (16.345%), Socio-economicfactor (10.626%), Experience factor (9.243%) and Constraints factor (7.506%) were isolated. Further results showed that household size ($r = 0.224$; $p \leq 0.01$) and years of formal education ($r = 0.211$; $p \leq 0.01$) had positive and significant relationship with effectiveness of the YCAD programme. It was concluded from the study that YCAD was highly effective in employment generation, provision of incentives and creation of market for agricultural produce among the beneficiaries.

Keywords: *Effectiveness, rural youth, Youth Commercial Agricultural Development,YCAD.*

INTRODUCTION

A number of programmes have been introduced in the past to stimulate the interest of youth in agricultural production and processing.These activities serve as source of empowerment and employment opportunities for the youth, thereby alleviating

poverty and ensuring youth development. The Federal Government and State in realization of the importance of youth had initiated some agricultural development strategies in the past such as National Directorate of Employment (NDE), Better Life Programme, Fadamaprogramme, Agricultural Developments Programmes (ADPs), River Basin Development Authority (RBDA), National Agricultural Land Development Authority (NALDA), Green Revolution Programme (GR),Operation Feed the Nation (OFN) (Iwuchukwu and Igbokwe (2012). Akpan (2010) submitted that youth empowerment means involving young people in decision making processes on issues that affect them, as well as entrusting them with the knowledge and skills necessary for them to effectively and meaningfully participate in issues that concern their well-being. He further stated that Nigeria's government has attempted to stimulate youth's interest in agricultural production and processing since the late 1980s. According to Ogunmola (2013) andAyinde and Torimiro (2014), youth can be described as a group of young people between the ages of 18 to 40. They are known to be innocent but optimistic about life.Though youths have desirable qualities that can promote agriculture, most of them have strong apathy towards it (Adedoyin, 2005). The development of the agricultural sector of the Nigerian economy therefore depends on the rural youths because the larger percentage of them serves as linkage between the present and the future as well as labour reservoir (Muhammad-Lawal*et al.,* 2009). However, a number of researchers have reported that some of the programmes initiated for this course have failed in the past (Torimiro*et al.,* 2008; Gate, 2014; Ayinde *et al*., 2016Ayinde*et al.,* 2017).

Assessing the effectiveness of agricultural development programmes is an invaluable tool within the agricultural sector. Decision makers require evidence of the efficient and effective use of resources. It is an invaluable tool in this regard because it allows the sector to address challenges and shortcomings in order to improve activities and programmes (Frankel-Reed, 2008). Indicators of the effectiveness of programs generally focus on measuring the changes in outcomes that reflect the objectives of the program. Effectiveness has been defined as the extents to which objectives are achieved and the extent to which targeted problems are resolved. Also, it is the degree to which a purpose is achieved. Effectiveness is one of the characteristics of the agricultural extension programme that has received a great deal of attention from education researchers and workers.

Youth Commercial Agricultural Development programme (YCAD) was established in 2012 with the objectives to systematically incentivize youth into sustainable commercial agriculture, generate employment opportunities to potential young entrepreneurs, entrench an entrepreneurial market oriented, demand driven and commercially viable programme to be run under a youth cooperative agenda and to prepare independent role model entrepreneurial youth as showcase of how enterprising youth must work (Ogunmola, 2013). Youth Commercial Agricultural Development (YCAD) programme was designed to accelerate the process of agricultural commercialization in Ekiti-State, thus helping in increasing employment opportunities for youth as well as facilitating value addition of

specific agricultural products while guaranteeing food security and increasing internally generated revenue (IGR). Potential young entrepreneurs were empowered to become employed in commercial agricultural value chain activities. According to Ogunmola (2013), YCAD programme was introduced to provide financial incentive to encourage the youth to practice commercial agriculture. The programme was designed to create rapid employment for the youth through active participation in modern agricultural practices by raising the production efficiency and productivity of the beneficiaries so as to arrest the present declining state of the Nigeria agriculture and ban of importation of agricultural produce. The foregoing arouses the quest to assess the factors influencing the effectiveness of commercial agricultural development programme among these rural youths. The main objective of the study is to identify factors influencing the effectiveness of youth commercial agricultural development programme in Ekiti State, Nigeria, with a view to describe the socio-economic characteristics of the YCAD programme beneficiaries;identify type of enterprises in the YCAD programme; andisolate factors influencing the YCAD programme effectiveness.

MATERIAL AND METHOD

The study was conducted in Ekiti State, Nigeria. The state is located in south-western region of the country within coordinates 7°40'N 5015'E / 7.667°N 5.250°E with a land area of 6,353 km^2 and population of 2,737,186 (NPC, 2006), with population projection of 3,270,800 in 2016. Ekiti State was created on 1st October, 1996 out of Ondo State. Its capital is Ado Ekiti. Ekiti State covers the former twelve local government areas that made up the Ekiti Zone of old Ondo State.Ekiti State is bounded on the South by Ondo State, on the North by Kwara State, on the East by Kogi State and on the west by Osun State.Ekiti State has 16 local government Areas, three senatorial districts (North, South and Central) with six federal constituencies. Multistage sampling procedure was used to select respondents for the study. At the first stage, stratified random sampling technique was used to select 55 percent of the 315 beneficiaries that participated in the programme enterprises from the YCAD register. Therefore, 83 respondents were selected from 150 beneficiaries in arable crop enterprise, 47 respondents from 85 beneficiaries in livestock enterprise, 22 respondents from 40 beneficiaries in nursery tree crop enterprise and 22 respondents from 40 beneficiaries in aquaculture enterprise making a total of 174 respondents. At the second stage, individual beneficiary contact was explored through the use of cell phone to locate the respondents. A well-structured and validated interview schedule was used to collect quantitative data which were summarised with percentages, means and standard deviation while chi-square and correlation were used to draw inferences. Also, factor analysis was used to isolate factors influencing the programme effectiveness.

RESULTS AND DISCUSSION

Socio-economic characteristics of youth

Results in Table 1 show that the mean age of the respondents was 37±5 years, majority (76.4%) of the respondents were male, majority (83.9%) were married, mean household size was 5±2 persons, majority (88.5%) were Christians,their mean year of formal education was 15 ± 2 years with mean farming experience of 10±3 years.These findings revealed that the respondents are still in their active age based on Ogunmola (2013) categorization of youth as a group of people that are found within the age group of 18 to 40 years of age. Also, Oladeji*et al.* (2013) observed that it is generally believed that males are often more energetic and could readily be available for energy demanding jobs like production farming.

Table 1: Socio-economic Characteristics of the respondents(n = 174)

Variables	Frequency	Percentage	Mean	Std. Dev.
Age (years)				
20-30	19	10.9	37	5
31-40	120	69.0		
41-50	29	16.7		
Above 50	6	3.4		
Sex				
Male	133	76.4		
Female	41	23.6		
Marital Status				
Single	27	15.5		
Married	146	83.9		
Separated	1	0.6		
Household Size				
2-4	88	50.6	5	2
5-7	73	42.0		
8-10	13	7.5		
Religion				
Christianity	154	88.5		
Islam	18	10.3		
Traditional	2	1.1		
Formal Education (years)				
12-14	41	23.6	15	2
15-17	127	73.0		
18-20	6	3.4		

Source: Field Survey, 2018.

Income per month

Results in Figure 1 revealed that the mean monthly income earned by the respondents was ₦41,000.00 with standard deviation of ₦23,000. This value represents the average monthly income of the respondents on their farm activities altogether and translated to ₦492,000 annually. This finding might support the findings of Ayinde (2011) that income is a difficult characteristic to measure given the fact that most rural dwellers do not keep proper record of their income and coupled with the fact that sometimes they may deliberately refuse to disclose the amount they actually realized for fear of taxation and security reasons.

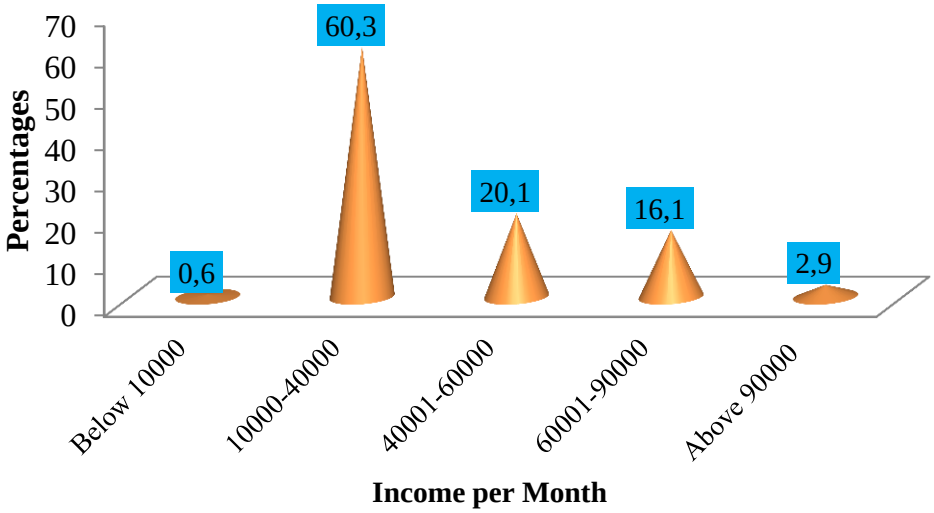

Fig. 1: Distribution of respondents based on their average income per month
Mean Score = ₦41,000
Standard deviation= ₦23,000
Source: Field Survey, 2018.

YCAD Enterprises

Results in Table 2 presents the identified enterprises in YCAD programme. It shows that Arable crop enterprises (47.7%) and Poultry (27%) were the most preferred enterprises by the beneficiaries in the study area followed by Tree crops enterprise (12.6%) and Aquaculture (12.6%) respectively. The implication of arable crop enterprise (47.7%) having the highest percentage of beneficiaries may be as a result of the fact that the nature of the soil in the study area is mostly conducive for arable crop farming.

Table 2. Enterprises in YCAD programme

Enterprises	Frequency	Percentage
Arable Crop	83	47.7
Poultry	47	27.0
Tree Crops	22	12.6
Aquaculture	22	12.6

Source: Field Survey, 2018.

Factors Influencing the Effectiveness of YCAD Programme

Table 3 shows the results of varimax factor rotation pattern with the measures that were highly loaded on each of the five factors extracted. Out of the 20 variables listed, the loading which give Eigen value of greater than one were five in number.Table 4 shows that the factors loaded explained 70.4 percent of variance in all while unknown factors explained the remaining 29.6 percent of the variance. The contribution of each of the highly loaded factors to influence the effectiveness of Youth Commercial Agricultural Development Programme were also shown as follows: Factor 1 –institutional support with 26.7percent contribution, followed by factor 2 – personnel (16.3%), factor 3 – economic (10.6%), factor 4 –experience (9.3%) andfactor 5 – constraints (7.5%).

Table 3. Result of varimax rotated component matrix showing extracted factors

Contributing Variables	1	2	3	4	5
Integrity of programme facilitators		0.801**			
Constraint variables					0.903**
Membership of agricultural association			0.672**		
Credibility of service provider		0.512**			
Source of information				0.759**	
Age			-0.342	0.767**	
Marital status				-0.456	
Provision of market by the government	0.555**				
Occupation		-0.774	0.673**		
Years of farming experience				0.857**	
Household size		-0.555	-0.431		-0.381
External orientation				0.440**	
Financial empowerment by government	0.744**				
Income per month			0.891**		-0.331
Years of formal education				0.783**	

Provision of agricultural inputs by government	0.864**	
Commitment of programme facilitators and service provider		0.556**
Competence of programme facilitators		0.860**
Good communication skill of programme facilitators		0.567**
Regular training of youth in different enterprises by government officials	0.602**	

**Loaded variables above 0.3

Source: Field Survey, 2018.

Table 4. Factor Names and percentage variation accounted for by each factor that Influences the Effectiveness of YCAD Programme

Component number	Factor label names	Eigen value	% variance	Cumulative%
1	Institutional support	4.834	26.672	26.672
2	Personnel	3.929	16.345	43.017
3	Socio-economics	1.806	10.626	53.643
4	Experience	1.971	9.243	62.886
5	Constraints	1.776	7.506	70.392
6-20	Other factors (not identified)	<1.000	29.208	100.00

Source: Field Survey, 2018.

Factor 1: Institutional support

This factor was defined by four measures of loading which were also positively loaded. These were provision of market (L=0.555), financial empowerment by government (L=0.744), regular training of youth in different enterprises by government (L= 0.602) and supply of agricultural inputs by government (L=0.864). The factor was named based on criterion three. The findings imply that support from various institutions will enhance the effectiveness of YCAD programme.

Factor 2: Personnel

This factor was identified by four measures which were positively loaded. These were integrity of programme facilitators (L=0.801), credibility of service provider (L=0.512), commitment of programme facilitators and service providers (L= 0.860) and good communication skill of programme facilitators (L=0.567). The factor was named based on criterion three. This implies that the better the

programme personnel characteristics, the better equipped they are to give their best for the success of the programme; they are also the ultimate means through which all other resources needed for the programme are acquired and allocated to the beneficiaries. The findings corroborate the submission of Adeloye (2016) who found that personnel characteristics influenced programme effectiveness.

Factor 3: Socio-economics

This factor was defined by five measures of loading out of which three were positively loaded. These were membership of agricultural association (L=0.672), occupation (L=0.673) and income (L=0.891).This factor was named based on criterion one. It implies that income realized from the programme can determine youths' involvement in YCAD programme because the amount of income benefits realized could determine whether the youth would continue with the programme during implementation and after the programme has ended, thereby determining the sustainability and the effectiveness of the programme.

Factor 4: Experience

This factor was defined by four measures of loading that were all positively loaded. These were source of information (L=0.759), years of farming experience (L=0.857), external orientation (L=0.440) and years of formal education (L=0.783).This factor was named based on criteria one and two. The finding implies that involvement of youth in YCAD depends largely on level of experience of youth in different farming enterprises before introduction of YCAD programme which could influence its effectiveness.

Factor 5: Constraints

The factor was identified by three measures of loading out of which only constraints variables (L=0.903) was positively loaded. Criterion one was employed to name the factor. This implies that constraints such as political instability, conflict between service providers in terms of service rendered, delay in payment of service rendered by the government, high cost of production, political interferences, poor attitude of beneficiaries among others could have negative impact on the effectiveness of YCAD programme.

Table 5. Measures of loading of each of the five factors isolated and the percentage contribution on how each of them influences the effectiveness of YCAD programme

Contributing variables	**L**	**L^2**	**Λ**
1. Institutional support			
Provision of market	0.555	0.3080	
Financial empowerment by government	0.744	0.5535	
Regular training of youth in different enterprises by government	0.602	0.3624	
Supply of agricultural inputs by government	0.864	0.7464	1.9703

2. Personnel			
Integrity of programme facilitators	0.801	0.6416	
Credibility of service providers	0.512	0.2621	
Commitment of programme facilitators and service providers	0.860	0.7396	
Good communication skill of programme facilitators	0.567	0.3214	1.9641
3. Socio-economics			
Membership of agricultural association	0.672	0.4515	
Age	-0.342	0.1169	
Occupation	0.673	0.4529	
Household size	-0.431	0.1857	
Income	0.891	0.7938	2.0008
4. Experience			
Source of information	0.759	0.5760	
Years of farming experience	0.857	0.7344	
External orientation	0.440	0.1936	
Years of formal education	0.783	0.6130	2.117
5. Constraints			
Constraints variables	0.903	0.8154	
Household size	-0.381	0.1451	
Income	-0.331	0.1095	1.07

L= Loading for factors,
$\mathbf{L}^2$= Square of loading factors
λ = Latent root for the factor (summation of the square of loading)
Source: Field Survey, 2018.

CONCLUSIONS

In view of the findings from the study, YCAD programme was effective in employment generation, provision of incentives and creation of market for agricultural produce. However, it was less effective in agricultural production on a commercial scale. Also, the study showed that institutional support factor ranked the highest of all the factors isolated to influence the effectiveness of YCAD programme.

Therefore, it was suggested that effort should be made by stakeholders to establish YCAD beneficiaries fully into practical agriculture after the training programme while there should be provision of adequate facilities like land, inputs, machinery and so on for them to maximize the benefits of the acquired knowledge so as to enable them to carry out agricultural activities on a commercial scale.Effective policy measures for follow-up by the programme monitoring and evaluation teams; and the extension experts must be put in place to encourage the ex-trainees to continue in practicing what they have been trained for.

In conclusion, to enhance effective management of these enterprises, beneficiaries should properly operationalize the crucial factors isolated in the study in order to improve their livelihood standard.

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Original Scientific paper
10.7251/AGRENG1903139P
UDC 631.528.6

THE FAILURES OF GENETICALLY MODIFIED ORGANISMS (GMOS): RESISTANCE, REGULATION, AND REJECTION

John PAULL

University of Tasmania, Hobart, Tasmania, Australia
*Corresponding author: j.paull@utas.edu.au; john.paull@mail.com

ABSTRACT

Genetically modified organisms (GMOs) have been contentious for more than three decades. Only 24 countries grow GMOs commercially. Four countries (USA, Canada, Brazil and Argentina) account for 85% of the global GMO hectares. Four crops (soy, corn, cotton and canola) account for 99% of GM hectares. Despite the veneer of social validity that regulators cast, the GMO sector has failed to gain a social licence. Where GM labelling is required, food manufacturers avoid GM ingredients. GMOs have failed to gain price parity with their non-GM counterparts, and they attract price penalties. Segregation of GMOs and non-GMOs has failed (with a tolerance of 0.9% GM contamination in so-called non-GM canola). GM has failed the coexistence test with a GMO growers contaminating neighbouring farms. GMOs are a biosecurity fail, with test plots of GM canola planted in the late 1990s still monitored two decades later for rogue canola plants. Most GMO crops are glyphosate dependent. Glyphosate is globally subject to massive litigation claims and awards, and is implicated in the causation of multiple cancers. Mechanisms for compensating farms contaminated by GMOs are lacking. The GMO industry has taken no responsibility for contaminations. GMOs are a threat to the organic sector and the maintenance of certification and price premiums. Most countries (88%) do not grow GMO crops. This paper considers the global experience of GMOs and the Australian experience as a microcosm of the global experience and as a case study.

Keywords: *Genetically engineered crops, GM canola, GM cotton, Marsh v Baxter, glyphosate.*

INTRODUCTION

The global adoption of genetically modified organisms (GMOs) has been limited despite three decades of robust marketing. Four countries of North and South America account for 85% of global GMO hectares: USA (40%), Brazil, (26%) Argentina (12%), and Canada (7%). Another 20 countries have some GMO crops. The global total of GMO hectares is 189.8 million hectares. (ISAAA, 2017a). Most countries (88%) have no GMO hectares.

Globally, four GMO crops account for almost all (>99%) of the world's GM crops: soy (50%), corn (31%), cotton (13%), and canola (5%) (ISAAA, 2017a). Two of

these 'big four' commercial GM crops, cotton and canola, are grown in Australia (OGTR, 2018b). Most countries (n=17) with commercial GMO grow just one or two GM crops (OGTR, 2018b). Any GM crop in Australia must be first approved by the Office of the Gene Technology Regulator (OGTR), based in Canberra. As elsewhere, there has been a bifurcation of views. GMOs have, from the outset, met with scepticism and rejection by Australian consumers, while being embraced and promoted by Australian university agriculture departments and the CSIRO (e.g. OGTR, 2019). This disparity of views persists to the present time. The clash of views is perhaps a part of the modern trend, borne of recent experience, to distrust experts (Shaw, 2016). The present paper examines the global experience of GMOs, it draws on the Australian experience as a case study, and it reveals multiple facets of the failures of the GMO farming sector.

MATERIAL AND METHODS

The present paper draws on multiple sources, including surveys of consumer attitudes over the past decade, longitudinal price data of GMOs, longitudinal plantings data of GMOs, legal trial and appeal documents, including evidence and judgements in the Marsh v Baxter case (where an organic grower, Marsh, sued a neighbouring GMO grower, Baxter, for economic losses, including loss of organics premium, due to loss of organics certification caused by GM contamination), and documentation (including submissions, hearings transcripts, and the official report) of the Parliamentary Inquiry into mechanisms for compensation for economic loss to farmers in Western Australia due to contamination by genetically modified material.

RESULTS AND DISCUSSION

Australia offers a microcosm of the global experience of GMOs. It is minor player in the world of GMO agriculture, and it accounts for 0.4% of the world's GMO agriculture (ABCA, 2019; Cotton Australia, 2019; ISAAA, 2017b). There are two GM crops commercially grown in Australia, GM canola and GM cotton (ISAAA, 2018). GMO agriculture in Australia accounts for 0.2% of Australian agricultural hectares (492,000 ha of GM canola plus 282,000 ha of GM cotton = a total 774,000 ha of GM crops, compared to a total 394,000,000 ha of Australian agriculture land) (ABCA, 2019; ABS, 2018; Cotton Australia, 2019). Given the experience of the past two decades, there appears to be little prospect of those GMO hectares increasing in the immediate future. Ten facets, including social, economic, agronomic, commercial and ecological aspects, of the failures of the GMO sector are documented.

Social license failure

Consumers of the world avoid GM foods. A multi-national study of consumers (n=23,000) across 17 countries, reported that 60% of Chinese consumers reject GM food, for Mexico and Italy the figure is 49%, and for Spain, Russia, France, and Brazil the figure is 45% (GfK, 2017).

As elsewhere, the results of community surveys conducted in Australia over more

than a decade reveal that GM food and crops have failed to achieve a social licence. There is no majority support for GMO food in Australia. In one survey (n=1,100), 66% of respondents were either "concerned" (39%) or "alarmed" (27%) about "Genetically modified GM foods", with a further 7% responding as either "Neutral" (4%) or "Don't know" (3%), and only 28% responding that they were either "excited (9%) or "hopeful (19%) (MARS, 2011). These results reveal community disdain for GMO foods and are consistent with previous similar surveys (e.g. MARS, 2008, n=1,100).

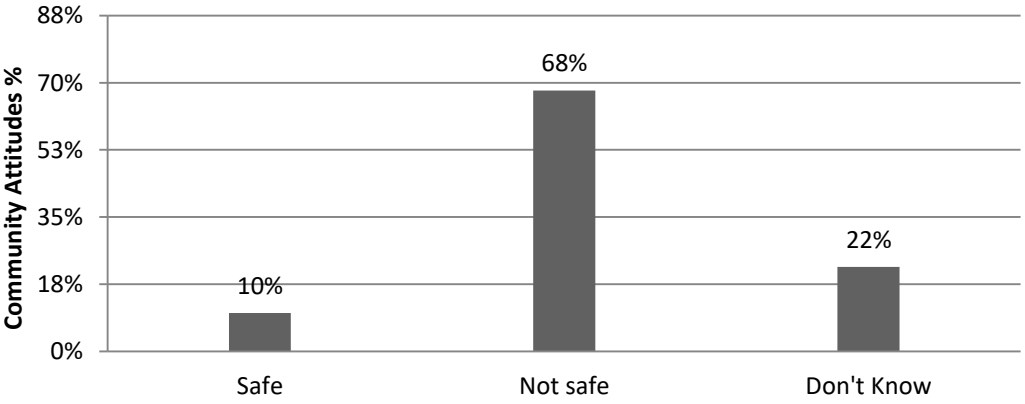


Figure 1. Australian community attitudes to "the use of genetically modified (GM) technology to produce food" (n=1,255) (author's graph; data source: Cormick & Mercer, 2017).

A survey commissioned by the OGTR (n=1,255), reported a minority (38%) of the community supported GM food and crops. This was consistent with 34% of respondents reporting their "willingness to eat GM foods". Only 10% of respondents reported that it was "safe" to grow GM in their own state or territory while 68% of respondents reported that it was "not safe" to grow GM in their own state or territory (Cormick & Mercer, 2017) (Fig. 1).

Retail failure

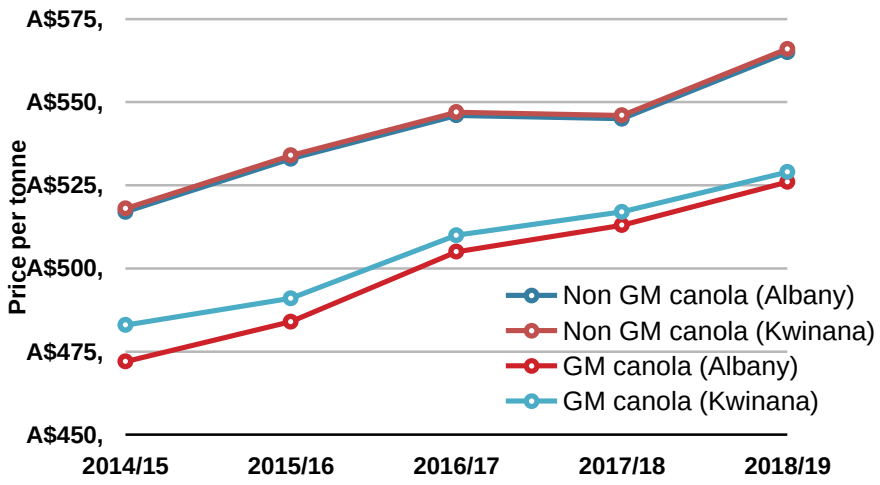
Where manufacturers are required to declare GMO ingredients in their products, they opt to avoid such ingredients**.** For example, GMO food products are required to be labelled as such in Australia (FSANZ, 2016). The consequence of this labelling requirement is that there are no GMO food products offered for sale in Australian supermarkets. The food processors, suppliers, and supermarkets have collectively and clearly made the judgement that Australian consumers have no appetite for GM foodstuffs in their diet, and that the inclusion of GM ingredients would damage their brand. Some products, for example Zafarelli pasta products, state on their packaging "Free of Genetic Modification" (Zafarelli pasta is Australian made from Australian grown durum wheat). GMO food is absent from Australian supermarket shelves and this outcome reflects (a) the food regulation

requirement to label GMO ingredients on the pack, (b) the resistance of Australians consumers to buy such products, and (c) the recognition, in the marketplace, of the prevailing negative consumer sentiments regarding GMOs.

Price failure

In the global market place, GMO crops attract a price penalty, in the region of 10% to 25% for GM soy (Açıkgöz, 2018). By choice or circumstance, the GM sector operates a 'sell cheap' price regime. Based on five seasons of data and two delivery depots, the price penalty for GM canola in Western Australia is 7.2%. Over the seasons and across the depots, the annual price penalty for GM varied from a low of 5.3% to a high of 9.2% (Paull, 2019b) (Fig. 2). About 21% of Australian grown canola is GM (OGTR, 2018b). For GM cotton, no price comparison is available because over 99% of Australia's cotton is GM (Cotton Australia, 2018).

Figure 2. Average annual price per tonne of GM canola versus non GM canola



(graph source: after Paull, 2019b; data source: Taylor, 2019).

Biosecurity failure

Once introduced into an environment, GMOs are challenging to eliminate or contain (Agapito-Tenfen et al., 2017). Australia's island state, Tasmania, has the strictest biosecurity regime of all Australian states and territories. There has been a GMO Moratorium in place fin Tasmania since 2001 and this persists to the present time (DPIPWE, 2019).

In the late 1990s to 2000, Monsanto and Aventis conducted field trials of GM canola at 57 sites in Tasmania. The sites have been monitored for the past two decades with multiple audits. Every audit has identified rogue canola plants, despite containment practices, with the number of plants declining (DPIPWE, 2014; Paull, 2019d) (Fig. 3).

These unwelcome GMO intruders into the Tasmanian landscape appear to be contained to the original trial sites, but not eliminated, even after two decades of auditing and containment practices. The data from the Tasmanian experience show

the persistence of GMOs in the landscape and the serious challenge of eliminating them once introduced, even in the circumstance here of limited experimental field trials which fall well short of commercial release.

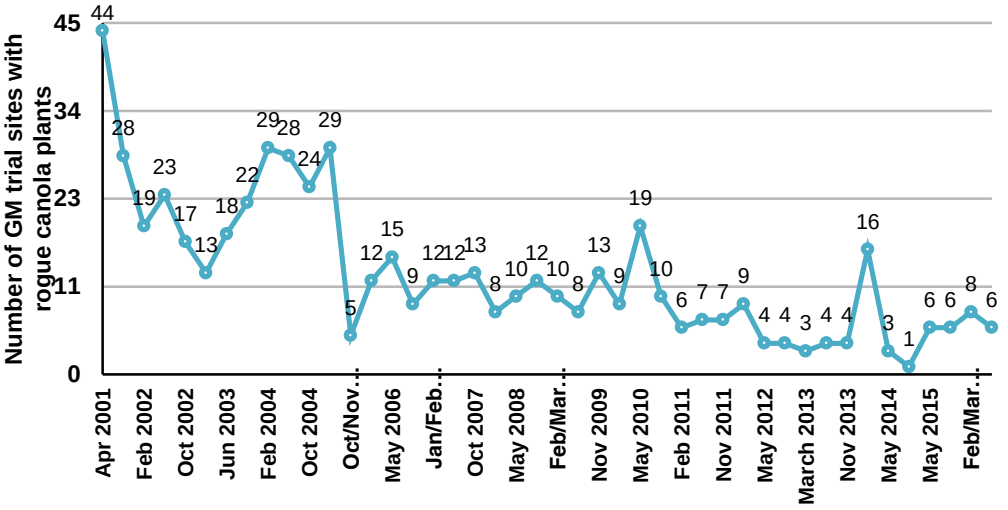


Figure 3. Monitoring of the 1990s GMO trial sites continues to find rogue canola plants at GMO trial sites in Tasmania even after two decades.

Segregation failure

Contamination by GMOs of their non-GMO analogues is a global phenomenon (Price & Cotter, 2014; Sharratt & Chopra, 2019). Importing countries, including China and South Korea, have rejected shipments of produce, including wheat and corn, due to GMO contamination (Chung, 2016; Lopez, 2013).

Table 1. The two classes of canola offered in WA as described by the grain handler (neither are without GMOs), with a 'fair description' added by the present author (CBH, 2019).

Canola Grade	Marketing Description	Specified Characteristics	Fair Description
CAN	"Non GM Canola"	"Certified GM free to Maximum adventitious presence of 0.9% GMO. Suitable for Human Consumption and Biodiesel production. ISCC EU Certified".	**Canola with GM contamination ≤ 0.9%**
CAG	"Canola"	"Suitable for Human Consumption and Biodiesel production. ISCC EU Certified".	**GM Canola**

When an exemption was made to the GM moratorium in Western Australia (WA) to allow GM canola in 2010 (Paull, 2015b), the exemption was made on the

assurance that GM and non-GM grain could coexist and a grain-handling segregation regime could and would avoid contamination of non-GM grain by GM grain. This promised segregation has been a failure. The assurances of strict segregation promptly failed as the impracticability of strict and effective segregation was revealed in practice. The outcome for WA (a state of 2.5 million km^2, larger than France, Spain, Germany, Portugal, Poland, Italy and UK taken together) is that canola exported from WA is graded as 'CAG' (= GM canola) or 'CAN' (= non-GM canola but with an allowed contamination by GM canola of up to 0.9%) (CBH, 2019). So, although the 'CAN' grade is described as 'non GM' by the grain handler, it is not GM-free (and is not non-GM in the usual usage of the language) (Paull, 2019b) (Table 1). This history of ongoing GMO segregation failures, in WA along with the biosecurity issues of the persistence of rogue canola plants in Tasmania from GMO trials of two decades ago, supports the case that GMOs are properly regarded and managed as invasive species (Paull, 2018a).

Stability failure

The number of countries growing GMOs is shrinking. It appears to have peaked at 29 countries in 2010, and from there its has shrunk progressively, to 28 in 2012, 26 in 2016 and to 24 presently (ISAAA, 2010, 2012, 2018).

The second GM crop that is grown in Australia is GM cotton, and most (>99%) of Australia's cotton production is GM cotton (Cotton Australia, 2018). Cotton growing is Australia has always been controversial for a variety of reasons, including that cotton is a 'water hungry' crop and Australia is a dry continent where water is precious and droughts are regular, the crop is grown as a broad-acre monoculture and relies on 'crop dusting' planes to apply a smorgasbord of biocides, with the attendant spray drift contaminations exacerbated by the aerial application of these toxic chemicals, and with the attendant contentious contamination of waterways. GM cotton has been grown in Australia since 1996 (OGTR, 2018a). In the period since then, the areas sown have varied wildly from year to year (Figure 4). Current plantings of cotton in Australia are less than they were in 1996/97. The industry does not exhibit any stability but instead exhibits erratic fluctuations (from a high of 599,630 ha in 2010, to a low of 68,585 ha in 2007, to presently 282,000 ha in 2018) (Fig. 4).

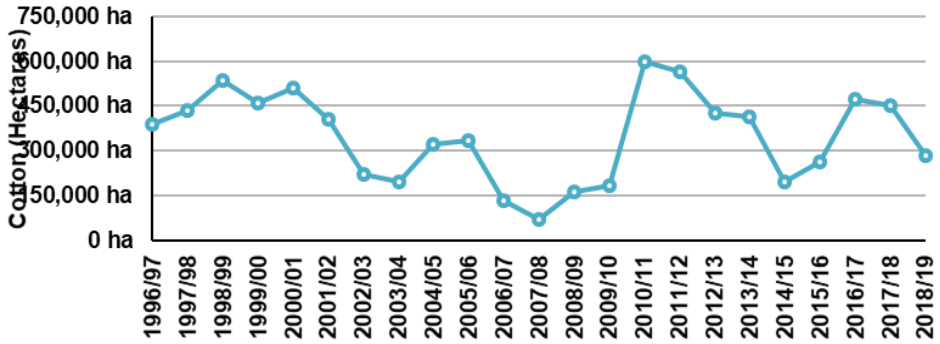


Figure 4. Cotton plantings in Australia have been erratic (author's graph; data source: Cotton Australia, 2019).

Coexistence failure

GMO farms have not proved to be 'good neighbours' to non-GMO farms (CBC News, 2004; McLachlin et al., 2001). GM canola was approved for release in WA in 2010 with the assurance that GM and non-GM cops could coexist. This immediately proved to be false. That first GM canola crop in WA contaminated a neighbouring organic farm, which, as a consequence, lost its organic certification. The representation had been made at the time of the initial approval that if there were contaminations that there were adequate common law remedies to recover losses. When put to the test, this assurance ultimately proved to be false (Paull, 2015b). The organic farmer (Marsh) sued the GMO farmer (Baxter) for the economic loss caused by the loss of the organic premium. The loss was agreed between the parties at A$85,000 (US$60,000; €53,000). The liability (but not the actual contamination) was contested by the GMO farmer at every legal step, as the case moved from the WA Supreme Court, to the WA Court of Appeal, and finally to the highest court in the land, the High Court of Australia. The legal costs of the GMO farmer were paid by Monsanto. The organic farmer lost the case at every step, and costs were awarded against the organic farmer.

The Marsh v Baxter case established that (a) GM crops can contaminate a neighbouring farm with impunity (the facts of the contamination were not contested although the judge preferred to characterise the offending events as 'incursions' rather than 'contamination') (Martin, 2014), that (b) there are no effective common law remedies for such contamination, and (c) a farmer suing a neighbour for losses due to GM contamination faces years of litigation (in this case, all fruitless), and risks bankruptcy, since the legal costs of the Marsh v Baxter case exceeded A$,2,000,000 (US$1,410,000; €1,250,000) (Paull, 2015a, 2015b).

Contamination failure

Non-GMO farmers bear the burden of GMO on-farm contaminations, and there are no ready solutions to this iniquity. Following a change of government in WA in 2017 (to Labor), there was the acknowledgement that the Marsh v Baxter case demonstrated that the common law remedies for GM contamination were deficient.

The WA Legislative Council (upper house of the WA bicameral parliament) established a Parliamentary Inquiry to consider "mechanisms for compensation for economic loss to farmers in Western Australia caused by contamination by genetically modified material" (EPAC, 2018). Public submissions were called for, and evidence was admitted in a series of hearings. The report of the Inquiry was disappointing. Various mechanisms were submitted and considered by the committee but none were recommended for implementation (Swinbourn, 2019) (Table 2). The committee was unable to determine the extent of contamination events occurring across the state .Members observed that the Marsh v Baxter case had had a "chilling effect" (e.g. Collins, 2018, p.4; May, 2018, p.9; Paull, 2018b, p.6) in silencing farmers experiencing GMO contamination because litigation had proved so expensive in terms of time and money in the Marsh v Baxter case and had ultimately only achieved further penalisation of the contaminated party.

Table 2. Options for a compensation mechanism considered (but not progressed) by the WA Parliamentary Inquiry (Paull, 2019a).

#	Proposed mechanism	Result
A	Status quo, i.e. Do nothing	The recommended outcome in the Inquiry Report
B	Levy GM industry	Not a recommendation in the Inquiry Report
C	Technology Licence Bond	Absent in the Inquiry Report
D	Non-GM farmer Insurance	Not readily available (or at all) in the marketplace
E	GM farmer Insurance	Not readily available (or at all) in the marketplace
F	Compulsory Third Party (CTP)	Not a recommendation in the Inquiry Report
G	Government pays	Absent in the Inquiry Report

The Parliamentary Inquiry was the last 'great hope' that some good, for the non-GM farming sector, might come from the WA experience of GM contamination and the Marsh v Baxter case. No such happy outcome was achieved. A GMO-contaminated party faces the prospect of no proven common law remedy and the might of Monsanto's purse (which backed GMO farmer Baxter in court and indemnified his risk). It would be a brave and perhaps foolhardy Australian farmer who next takes on Monsanto/Bayer GM contamination in the light of the Marsh v Baxter case which has now run its course and exhausted its legal options.

Uptake failure

GMOs account for 3.8% of the world's agricultural land and although this has been creeping up, the adopter base has been shrinking. Globally, the number of countries planting GMOs peaked in 2010 (n=29) and has been declining since then (currently n=24) (ISAAA, 2010, 2018). Australia's OGTR (established in 2000) has approved

the commercial release of GM cotton since 2002 and GM canola since 2003 (OGTR, 2019), nevertheless GMOs only account for less than 0.2% of Australian agriculture hectares (Fig. 5).

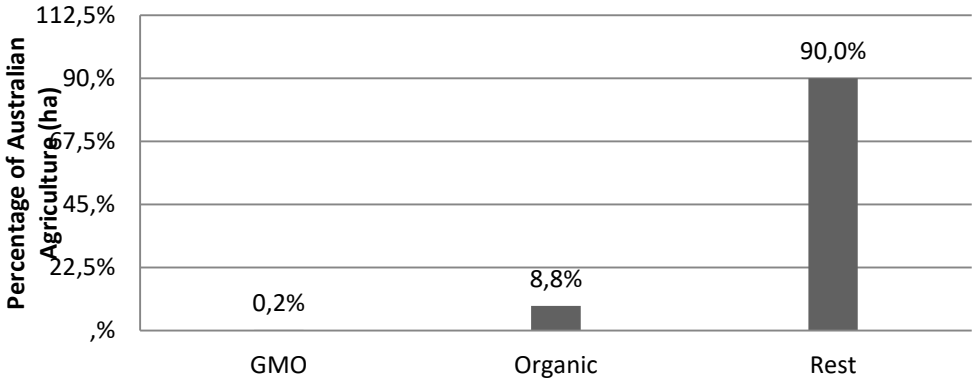


Figure 5. The distribution of three mutually exclusive types of agriculture in Australia.

The percentage of the canola crop in WA that is GM canola has been in decline since 2015 (Fig. 6). The most recent figures reveal that GM canola planting has declined while total canola plantings have increased: "The proportion of canola sown to Roundup Ready varieties contracted to 18% of the area. This was related to the increase in canola area (from 1.2 in 2016 to 1.4 million Ha in 2017) … and a small decrease in the area of GM canola sown" (Bucat, 2018, p.5).

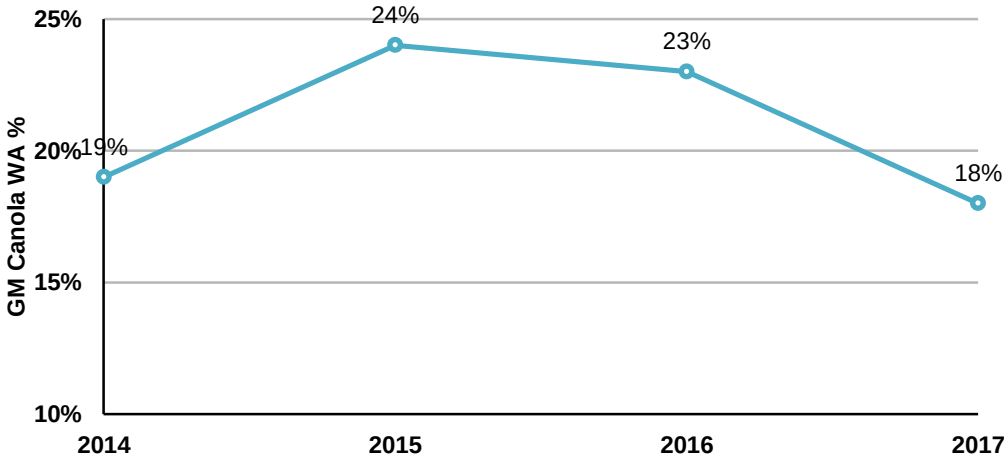


Figure 6. Percentage of the WA canola crop that is GM canola.

Glyphosate failure

Most of the world's GMOs are herbicide tolerant, with variations of Monsanto's GM Roundup Ready (RR) soy, corn, cotton and canola the most popular (ISAAA,

2018). The GM canola crop in Australia is a glyphosate dependent crop (Monsanto's RR canola). Glyphosate is a problematic herbicide, it is a declared carcinogen (OEHHA, 2019), it is ingested by adults and children via a variety of routes including food and beverages (Cook, 2019), and it is currently under consideration of being banned in multiple jurisdictions.

There have recently been several landmark decisions awarding damages for cancer caused by glyphosate, in one case, US$80 million (Rosenblatt, 2019), in another, US$289 million (Bellon, 2018), and in another, US$2 billion (Davis, 2019). There are a further 9,300 more plaintiffs in USA (Bender, 2018). The first lawsuit has been filed in Australia, with more forthcoming (Houston & Vedelego, 2019). This is a global problem for Monsanto and its new owner Bayer.

The case for GM canola (RR canola) has been built on foundations that are in the process of being swept away in a blizzard of litigation. Glyphosate is a cancer causing herbicide that is a faltering cornerstone for the GM industry to have staked so much, including reputation, in Australia and elsewhere.

CONCLUSION

The GMO industry has failed major tests, including, the lack of social licence, attracting price penalties, lapses of biosecurity, segregation, stability, coexistence, contamination, narrow uptake base and market penetration, and glyphosate dependence. Australia offers a microcosm for considering these failures. Australia is a major player in global agriculture and in global agriculture exports (Rural Bank, 2018), but it is a minor player in the world of GMOs. In line with global consumers, the Australian public have failed to concede a social licence to this industry and remain skeptical about GMOs. The GM hectares in Australia are in decline for the two GM crops, GM canola and GM cotton. There is a price penalty for GM canola of 7.2% compared to non GM canola (no comparison figures are available for GM cotton versus non GM cotton). GM canola is a glyphosate dependent crop and its percentage of the canola crop in Australia may be anticipated to plummet now that cancer lawsuits are in propect, if glyphosate use is banned, and if glyphosate residues are implemented at zero-tolerance by the market.

In contrast to the declining GM sector, the organic sector in Australia, and the world, is in the ascent and Australian organics now accounts for 51% of the world's certified organic hectares (Paull, 2019c). Australia is far from the world's loci of pollution, and has many other natural advantages for 'clean and green' food and fibre production. GMO farming puts at risk 'Brand Australia' as a clean and green source of premium food and fibre. Extrapolating from present trends, we may foresee GMO production further retreating in Australia, as resistance is maintained within Australia and rejection is entrenched and increasing internationally as discerning markets and consumers say 'no' to GMO imports.

ACKNOWLEDGEMENT

A version of this paper was presented as a keynote address at AGROSYM 2019 at

Johorina. That presentation is available at: http://orgprints.org/36573/1/Paull.2019.AgrosymKeynote.GMOs.pdf and at academia.edu.

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Original Scientific paper
10.7251/AGRENG1903153K
UDC 634.8:631.811.98(560)

EFFECTS OF SHOOT TRIMMING AND ETHEPHON TREATMENTS ON VEGETATIVE CHARACTERISTICS OF 'USLU' GRAPEVINE

Önder KAMILOĞLU*, A. Aytekin POLAT

Mustafa Kemal University, Faculty of Agriculture, Department of Horticulture, Hatay, Turkey
*Corresponding author: okoglu@gmail.com

ABSTRACT

Mediterranean, depending on the ecological conditions, is an important region in terms of earliness and grape cultivation in Turkey. Vegetative growth control is deemed to be necessary in grape cultivars that exhibit strong growth. This experiment was carried out for grapevines of Uslu grafted onto 1103 P rootstock at a spacing of 2 m × 3 m to analyze the influence of ethephon and shoot trimming on vine growth characteristics. Shoot trimming (control and 10 nodes) and ethephon (0, 500, 750 ppm) applications were carried out on vinestocks approximately one week before blooming. Summer shoot length (cm), node number of summer shoot (n), internode length of summer shoot (cm), axillary bud burst percentage (%), number of axillary shoot (n), mean length of axillary shoot (cm), total length of axillary shoot (cm), cane diameter (mm), pith diameter (mm), cane/pith, pruning wood weight (g) were determined. According to results, foliar treatments with ethephon applied in shoots growth period (before flowering) exhibited a strong inhibitory effect on shoot growth of cv. Uslu. Ethephon (500 ppm and 750 ppm) application reduced summer shoot length, node number and internode length. Increasing ethephon doses reduced active bud burst percentage, shoot length and pruning wood weight. The number of axillary shoots decreased by 49.4% in trimmed plants in comparison with the control. It was determined that mean length of axillary shoot was higher in trimmed plants, while total length of axillary shoot and pruning wood weights of grapevines were similar in both trimmed and control plants.

Key words: *Grapevine, summer shoot, axillary bud, ethephon, trimming, vegetative growth*

INTRODUCTION

Turkey is an important table grape producer. Table grapes are grown in 50.2% of the vineyards within the country. In terms of ecological conditions, table grapes ripen initially in Mediterranean region. The Mediterranean Region has an important role in grape production, producing 715.781 tons in 79.468 ha of

vineyard (TUIK, 2017). In addition to ecological advantages of the region for earliness, selection of cultivars is also a very important factor in terms of ripeness time. Depending on these two factors, early harvested table grapes can be easily marketed at a high price. In this regard, the earliest grape cultivar in the region is 'Uslu'. This cultivar is bred in Turkey, seeded, dark red, and it has large berries (Celik, 2006). Its yield is average, and its grapevine are vegetatively strong (Tangolar et al., 1996). Shoot development of the cultivar is faster than other cultivars in both greenhouse and open cultivation (Kamiloglu et al., 2011). This cultivar has the tendency to produce numerous and strong axillary shoots (Turker and Dardeniz, 2014).

Intensive growth of vines in warm climates requires measures to control vigour in order to ensure fruit quality and vegetative balance of the plants (Di Lorenzo et al., 2011). In addition, in grapevines the amount of fruit is mainly governed by the number of shoots and not by their length (Lavee, 2000). The traditional method of containing excessive vegetative growth is to stop the vines by removing a length of the terminal part of the cane. But this practice suffer from several important disadvantages: its effect is limited in duration, axillary bud growth is promoted and the proportion all dry matter production allocated to such dispensible portions of growth as lateral shoot may even be increased (Nickel, 1983).

Certain growth regulators are used in keeping shoot development under control. Plant growth retardants used in viticulture for modification of fruit setting affect shoot development as well. The control of excessive vigor offers a number of advantages. Shading of the fruit is reduced, cultural practices are facilitated and the competitive sink strength of the developing shoot tips is reduced (Szyjewicz et al., 1984). Ethephon is an inhibitor effective in keeping vegetative development under control. When compared with the other growth regulators, ethephon is highly effective ethylene generator which most effectively controls excessive vigor on various cultivars (Szyjewicz et al., 1984). In a study conducted for this purpose, it was reported that 420-720 mg/L doses yielded the best result in terms of inhibiting shoot development, and ethephon also prevented growth comprising lateral buds (Shulman et al.,1980; Polat, 2002).

In this experiment, the effects of shoot trimming and different ethephon dose applications before blooming, intended for keeping vegetative development under control in Uslu grape cultivar, was researched.

MATERIAL AND METHODS

The experiment was carried out in Dörtyol city, in Eastern Mediterranean of Turkey. The experiment area of Agriculture Faculty of Hatay Mustafa Kemal University is located at 36°54´N and 36°13´E at 198 m.a.s.l. It has subtropical climate and the yearly average temperature is 19.0 °C, with 886 mm precipitation, which primarily falls during winter and spring. Eight-year-old *Vitis vinifera* L. cv. 'Uslu' (Hönüsü × Siyah Gemre), grafted onto '1103 P' (Berlandieri X Riparia) rootstock grapevines were used for this study. The vines are established at 3 × 2 m intervals and trained using bilateral cordon system. All practices were carried out

1st year and 2nd year on May 04 and May 05 respectively, approximately one week before blooming. Ethephon and trimming applications were experimented in the study. Shoot trimming was done by hand to maintain at 10 nodes per summer shoot. Grapevines with or without trimming were applied ethephon 0, 500, 750 ppm doses. Ethephon was sprayed to the upper part of primary shoots after clusters.

In order to determine their effects on vegetative developments of grapevine, certain measurements were made immediately after applications (at the beginning of the experiment) and 30 days later, and during dormant season. Four summer shoots were marked on each grapevine that was trained in bilateral cordon trellis. Eight summer shoots were used for each repetition. Immediately after treatment, shoot length in control (untrimmed) and trimmed vines were 119 and 76 cm, respectively. Likewise, total axillary shoot lengths were respectively 57 and 60 cm.

In the experiment; summer shoot length, summer shoot node number, summer shoot internode length, axillary bud burst percentage, number of axillary shoot, mean length of axillary shoot, total length of axillary shoot (cm) were determined. Pruning wood weights of the grapevines were weight in the dormant season. In the marked canes, two-way measurements were made from the midpoint between the internodes of the nodes 4-5. and 5-6. The cane and pith diameters were recorded in the measurements. The cane/pith ratio was calculated (Dardeniz and Şahin, 2005). Length measurements were made with measuring tape, diameter measurements were made with caliper, weight measurements were made with 1 g electronic precision scale, and counts were taken visually and by hand.

The experiment was arranged in a Randomized Complete Blocks with three replications. There were two vine per replication. Variance analysis was carried out through MSTAT statistical software and means were compared by Tukey Test (α=0.05; 0.01).

RESULTS AND DISCUSSION

The grapevine is generally considered a vigorous species developing long and rapidly growing canes (Patterson and Zoecklein, 1990). Significant increases can be seen in vine growth particularly due to ecological conditions and cultural treatments (watering, fertilization). It is attempted to keep vegetative development of vines under control by means of summer and winter pruning and, if deemed necessary, the use of certain plant growth retardants. In this study, effects of trimming and ethephon applications on vine development under subtropical climate conditions was researched.

During the study, the effect of applications on summer shoot length was found to be significant at a level of 1% in terms of the mean values of each year and both years. Similarly, 500 ppm and 750 ppm ethephon treatments statistically reduced shoot growth in comparison with the control. In addition, it was seen that trimming significantly limited shoot length. Upon examination of treatment x dose interaction, it was determined that ethephon doses did not affect shoot development in trimmed plants, while 500 ppm and 750 ppm doses reduced shoot development

in plants that were not trimmed in comparison with the control (Table 1). According to the summer shoot measurements at the beginning of the experiment and repeated in 30th day, (Figure 1) level of development was proportionately 95.6% in comparison with the control (0 ppm), 25% at 500 ppm, and 21% at 750 ppm in plants that were not trimmed. This increase was maximum 2% in grapevines that were trimmed.

While the effect of ethephon doses on the number of summer shoot nodes vary according to years, the mean value of both years was found to be significant at 5%. In comparison with 0 ppm, other two doses reduced the number of nodes. In trimming application, reduction of nodes in comparison with the control was an expected result (Table 1). According to mean values of the 2nd year and the both years of the study, mean internode length of summer shoots was reduced in 500 ppm and 750 ppm doses, and this effect was found to be statistically significant. Internode length of summer shoots varied according to years in plants that were trimmed and not trimmed (Table 1). It was determined that the number of axillary shoots, formed on nodes of summer shoots, were lower in trimmed plants than the control and lower in 750 ppm ethephon dose than the other doses according to the mean values of two years. In treatment x dose interaction, while trimmed plants were statistically affected in a similar manner from ethephon doses, control treatment exhibited variance based on doses (Table 2).

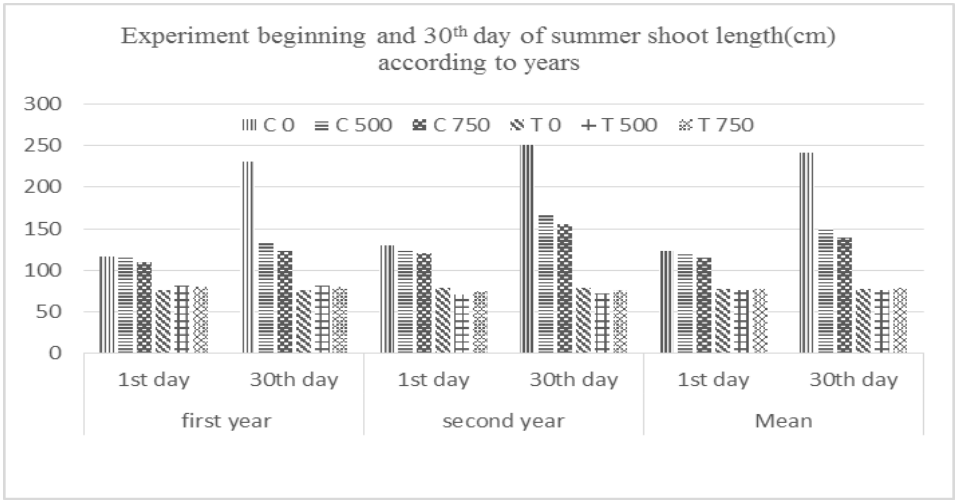

Figure 1. Experiment beginning and 30th day of summer shoot length (cm) according to years

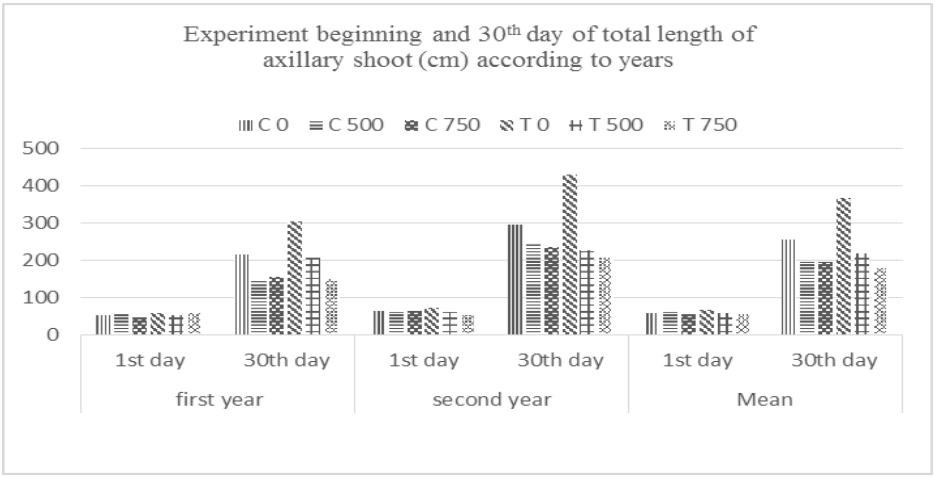

Figure 2. Experiment beginning and 30th day of total length of axillary shoot (cm) according to years

Table 3. Effects of trimming and different ethephon doses on cane growth and pruning wood weight of 'Uslu' table grape

Treatment	Dose (ppm)	Cane diameter (mm)			Pith diameter (mm)			Cane/pith diameter			Pruning wood weight (g)		
		1st year	2nd year	Mean	1st year	2nd year	Mean	1st year	2nd year	Mean	1st year	2nd year	Mean
Control	0	8.93	8.97	8.95	3.67	4.09	3.88	2.55	2.28	2.41	2900.00	1528.00	2214.00
	500	7.65	8.91	8.28	3.21	4.23	3.72	2.48	2.20	2.34	1892.00	885.00	1388.50
	750	8.11	8.89	8.50	3.55	4.26	3.90	2.33	2.20	2.26	1824.50	1071.67	1448.08
Trimming	0	8.91	9.78	9.34	3.73	4.34	4.04	2.50	2.38	2.44	2322.17	1295.33	1808.75
	500	8.45	8.73	8.59	3.63	4.02	3.83	2.40	2.26	2.33	1795.83	777.50	1286.67
	750	7.77	8.69	8.23	3.65	4.31	3.98	2.23	2.11	2.17	1957.83	796.83	1377.33
Mean	Control	8.23	8.92	8.57	3.48	4.19	3.84	2.45	2.22	2.34	2205.50	1161.56 a	1683.53
	Trimming	8.38	9.06	8.72	3.67	4.23	3.95	2.38	2.25	2.31	2025.28	956.56 b	1490.92
Mean	0	8.92	9.37	9.14 a	3.70	4.22	3.96	2.53 a	2.33	2.43 a	2611.08 a	1411.67 A	2011.38 A
	500	8.05	8.82	8.44 b	3.42	4.13	3.77	2.44 ab	2.23	2.33 ab	1843.92 b	831.25 B	1337.58 B
	750	7.94	8.79	8.36 b	3.60	4.28	3.94	2.28 b	2.15	2.22 b	1891.17 b	934.25 B	1412.71 B
Dose		NS	NS	*	NS	NS	NS	*[1]	NS	*	*	**[1]	**
Treatment		NS	NS	NS	NS	NS	NS	NS[1]	NS	NS	NS	*	NS
Interaction		NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[1]NS, *, ** represent not significant and significant effect at the 0.05 (different lower case letter) and 0.01 (different capital letter) levels, respectively.

Table 1. Effects of trimming and different ethephon doses on summer shoot growth of 'Uslu' table grape

Treatment	Dose (ppm)	Summer shoot length (cm)			Node number of summer shoot (n)			Internode length of summer shoot (cm)		
		1st year	2nd year	Mean	1st year	2nd year	Mean	1st year	2nd year	Mean
Control	0	231.5 A	251.5 A	241.5 A	29.4 A	26.2	27.8 a	7.88 A	9.60 A	8.69 A
	500	133.5 B	168.0 B	150.8 B	21.3 B	22.4	21.9 b	6.27 AB	7.50 B	6.90 BC
	750	122.6 B	155.4 BC	139.0 B	20.5 B	21.3	20.9 b	5.97 B	7.30 B	6.65 C
Trimming	0	76.0 C	79.2 CD	77.6 C	10.0 C	10.0	10.0 c	7.60 AB	7.92 B	7.76 AB
	500	81.3 C	71.5 D	76.4 C	10.0 C	10.0	10.0 c	8.13 A	7.15 B	7.64 B
	750	80.5 C	76.5 CD	78.5 C	10.0 C	10.0	10.0 c	8.05 A	7.65 B	7.85 AB
Mean	Control	162.5 A	191.7 A	177.1 A	23.7 A	23.3 A	23.5 A	6.7 B	8.1 A	7.4 b
	Trimming	79.3 B	75.7 B	77.5 B	10.0 B	10.0 B	10.0 B	7.9 A	7.6 B	7.8 a
Mean	0	153.8 A	165.4 A	159.6 A	19.7 A	18.1	18.9 a	7.7	8.8 A	8.2 A
	500	107.4 B	119.8 AB	113.6 B	15.6 B	16.2	15.9 b	7.2	7.3 B	7.3 B
	750	101.6 B	116.0 B	108.8 B	15.3 B	15.6	15.5 b	7.0	7.5 B	7.2 B
Dose		**[1]	**	**	**	NS	*	NS[1]	**	**
Treatment		**	**	**	**	**	**	**[1]	**	*[1]
Interaction		**	**	**	**	NS	*	**	**	**

1 NS, *, ** represent not significant and significant effect at the 0.05 (different lower case letter) and 0.01 (different capital letter) levels, respectively.

Table 2. Effects of trimming and different ethephon doses on axillary shoot growth of 'Uslu' table grape

Treatment	Dose (ppm)	Axillary bud burst percentage			Number of axillary shoot			Mean length of axillary shoot			Total length of axillary shoot (cm)		
		1st year	2nd year	Mean	1st year	2nd year	Mean	1st year	2nd year	Mean	1st year	2nd year	Mean
Control	0	71.5 (57.7)	76.6 (61.1)	74.0 (59.4)	21.1A	20.0	20.6a	10.2b	14.6B	12.4B	216.3	295.5	255.9
	500	64.9 (53.7)	65.4 (54.0)	65.2 (53.9)	14.0B	15.0	14.5b	10.2b	15.5B	12.9B	148.6	242.9	195.8
	750	62.1 (52.0)	64.1 (53.3)	63.1 (52.7)	12.8BC	14.0	13.4b	11.9b	16.4B	14.2B	156.3	236.3	196.3
Trimming	0	84.2 (66.7)	81.3 (64.4)	82.7 (65.5)	8.4C	8.1	8.3c	36.4a	52.3A	44.3A	303.5	428.0	365.7
	500	85.8 (68.1)	80.8 (64.1)	83.3 (66.1)	8.6C	8.1	8.3c	24.5ab	28.1B	26.2A B	207.7	226.0	216.9
	750	82.5 (65.3)	77.1 (61.4)	79.8 (63.4)	8.3C	7.7	8.0c	18.4b	27.0B	22.6B	152.6	208.2	180.4
Mean	Control	66.2 (54.5)B	68.7 (56.1)B	67.4 (55.3)B	16.0A	16.4A	16.2A	10.8B	15.5B	13.2B	173.7 b[1]	258.2	216.0
	Trimming	84.2 (66.7)A	79.7 (63.3)A	81.9 (65.0)A	8.4B	8.0B	8.2B	26.4A	35.8A	31.0A	221.3 a	287.4	254.3
Mean	0	77.8 (62.2)	78.9 (62.7)a	78.4 (62.5)a	14.8A	14.1	14.4A	23.3	33.5a	28.3a	259.9 A	361.7a	310.8A
	500	75.4 (60.9)	73.1 (59.1)ab	74.3 (60.0)ab	11.3B	11.6	11.4 AB	17.3	21.8b	19.6b	178.2AB	234.5b	206.3B
	750	72.3 (58.6)	70.6 (57.4)b	71.4 (58.0)b	10.5B	10.9	10.7B	15.1	21.7b	18.4b	154.4 B	222.3b	188.3B
Dose		NS[1]	*[1]	*	*	NS	**	**	NS	*	**	*	**
Treatment		**[1]	**	**	**	**	**	**	**	**	*	NS	NS
Interaction		NS	NS	NS	**	NS	*	**	*	**	NS[1]	NS	NS

[1]NS, *, ** represent not significant and significant effect at the 0.05 (different lower case letter) and 0.01 (different capital letter) levels, respectively. Values in parenthesis is angle transformation.

Patterson and Zoecklein (1990) reported that 750 ppm ethephon treatment decreased the number of laterals in vines, on which it was applied twice. In our study, it was seen that 500 ppm and 750 ppm doses exhibited reduction in comparison with control (0 ppm). As this characteristic was examined proportionally, a contrary situation was seen in terms of applications, while trimming application yielded a higher value than the control, and it was found to be significant at a level of 1% in terms of the mean value of years. Increasing ethephon doses caused a decrease in axillary bud burst and the lowest value was obtained from 750 ppm (Table 2). Gonzalez et. al. (2011) reported that 400, 800 $mg.L^{-1}$ ethephon treatments after berry set period significantly reduced sprouting of lateral buds, as well as leaf area development, leaf chlorophyll content and net photosynthesis rate in comparison with the control according to the measurements carried out on Verdejo vines in veraison period.

The effect of trimming and ethephon application on mean axillary shoot length was found to be statistically significant. While higher values were obtained in trimmed plants, 500 ppm and 750 ppm doses of ethephon caused a reduction in mean axillary shoot length (Table 2).

Total length of axillary shoots, formed by active budding in summer shoots, was higher in trimmed plants than untrimmed ones for the first year. It was determined that this effect continued for second year and the mean of both years; however, it was not statistically significant. A further reduction was seen in development of these shoots based on the increase in ethephon doses. However, the effect of 500 ppm and 750 ppm doses were statistically found to be similar (Table 2). Total axillary shoot lengths in summer shoots were found to be between 55.4 and 65.6 cm depending on the application and doses at the beginning of the experiment (Figure 2). In comparison with the beginning, mean axillary shoot length at 30^{th} day exhibited 102% to 134% proportional increase in plants that were not trimmed. The proportional increase in trimmed plants were 437% in control (0 ppm), 258% at 500 ppm, and 191% at 750 ppm.

The effect of trimming on cane diameter was not found to be significant in measurements made in the dormant season. Likewise, while ethephon did not exhibit an effect on diameter growth for each year, increasing ethephon dose reduced diameter growth according to the mean value for both years (Table 3). Pith layer, which is an indicator of lignification, was not affected by the application and ethephon doses in the experiment. Cane diameter/pith diameter exhibited a statistical change by years, while the dose of ethephon that increased in terms of the mean value of years partially reduced this value. In comparison with the control, trimming application did not exhibit any effect on pruning wood weight (except for the 2nd year). However, it was determined that ethephon doses were statistically significant at 5% and 1%, and that 500 ppm and 750 ppm doses reduced pruning wood (Table 3).

Patterson and Zoecklein, (1990) determined in their study that the application of 750 ppm ethephon dose once or twice to vines did not lead to a difference in

pruning wood weight, but other applications reduced pruning wood weight in comparison with the control. Mannini et al. (1981) in their study found that the topping treatment (1000 ppm ethephon) reduced the pruning weights relative to the 300 ppm ethephon treatments. However, Gonzalez et. al. (2011) reported that the reduction in lateral shoot development in plants treated with ethephon was compensated with further development of main shoot and total pruning wood remained unchanged. Weaver and Pool (1971) reported that particularly high doses weakened plant development in 'Thompson Seedless' vines treated with ethephon. It was reported that plant growth retardants temporarily delayed shoot growth during rapid growth periods of plants. A temporary delay in shoot growth during this period showed a positive effect on the fruit set (Todıć et al., 2012).

CONCLUSION

In the study, ethephon applied in shoot growth period (before flowering) exhibited a strong inhibitory effect on shoot growth of cv. Uslu. Ethephon (500 ppm and 750 ppm) application reduced summer shoot length, number of nodes and internode length. Increase ethephon doses reduced axillary bud burst percentage and pruning wood weight. Trimming significantly limited summer shoot growth. The number of axillary shoots was found to be lower in trimmed plants than the control and in ethephon 750 ppm dose than the other doses. While mean axillary shoot length was higher in trimmed plants, this effect was not seen in total axillary shoot length and pruning wood weights of grapevines were found to be similar.

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Original Scientific paper
10.7251/AGRENG1903164P
UDC 636.4:612.11(497)

HEMATOLOGICAL AND SERUM BIOCHEMICAL PROFILE IN EAST BALKAN PIGS AT DIFFERENT AGE AND SEASONS

Nadezhda PALOVA[1*], Yordan MARCHEV[2], Radka NEDEVA[2], Jivko NAKEV[2], Dimitrinka KRUSHEVA[1], Todor SLAVOV[3], Ivelina NEDEVA[3], Teodora POPOVA[4]

[1]Scientific Centre of Agriculture- Sredets, Bulgaria
[2]Agricultura Institute – Shumen, Bulgaria
[3]Trakia University – Stara Zagora, Bulgaria
[4]Institute of Animal Science- Kostinbrod, Bulgaria
*Corresponding author: nadejda_palova@abv.bg

ABSTRACT

Twelvehematological and fifteen serum biochemical parameters were determined in indigenous East Balkan pigs at different ages and two seasons. The experiment was carried out in the Scientific Centre of Agriculture- Sredets aiming to characterize the health status of the animals when reared organically. Blood samples were taken from pre-weaned piglets (n=10), growers (n=10) and sows (n=10) in spring and summer. The results were analysed through two way ANOVA to assess the influence of the age, season and their interaction on the hematological and serum biochemical profile of the animals. Both age and season interacted significantly in regard to the red blood cells count (RBC) ($P<0.001$), hemoglobin (HGB) ($P<0.001$) and hematocrit (HCT) ($P<0.01$), as well as in the most biochemical parameters including creatinine($P<0.001$), total protein (TP) ($P<0.001$), albumin (ALB) ($P<0.001$), alanine aminotransferase (ALT) ($P<0.001$), aspartate aminotransferase (AST) ($P<0.01$), uric acid (UA) ($P<0.01$), Mg ($P<0.001$), triglycerides (TG) ($P<0.05$) and cholesterol ($P<0.01$). Regardless of the season, the white blood cells (WBC) including lymphocytes and granulocytes, as well as platelets (PLT)decreased with age, while mean corpuscular volume (MCV) increased.The content of glucose, urea, gamma-glutamil transferase (GGT), alkaline phosphatase (ALP), Ca and P which displayed maximal values in pre-weaned pigs ($P<0.05$) compared to the other age groups ($P<0.05$). The season affected the number of lymphocytes ($P<0.01$), their percent ($P<0.001$), mean corpuscular hemoglobin (MCH) andmean corpuscular hemoglobin concentration (MCHC) ($P<0.001$), as well as the concentration of GGT ($P<0.001$) which increased during summer,and also granulocytes ($P<0.01$), their percentage ($P<0.001$), PLT ($P<0.05$), glucose, urea, ALP, Ca ($P<0.001$) and P ($P<0.01$) whichwere higher in spring.

Keywords: *Hematological and serum biochemical parameters, Pigs, East Balkan breed, Age, Seasons.*

INTRODUCTION

The study of the hematological and biochemical profile is an important method for monitoring of the health status in farm animals. It allows, on the one hand, to reveal the physiological-biochemical determination of some or other traits, and on the other, it is an internal indicator for prediction of the animal performance (Chemshirova, 2000). Although blood is known to be relatively persistent, it still remains one of the labile systems in the body. Comparing animals to haematological and serum biochemical parameters requires an accurate idea of the regularities in their variations. The main factors of affecting the variability of hematological and biochemical profile include animal age (Czech et al., 2017), level and type of nutrition (Fisher et al., 2013), environmental conditions (Mayengbam and Tolenkhomba, 2015) and hence these parameters can be a good indicator for determining the impact of different farming systems, food practices, seasonal and climatic changes on the health status of the animals. Indigenous pigs occupy an unique position in our animal genetic resource. East Balkan Pig is an indigenous domesticated pig breed in Bulgaria, distributed on the western coast of the Black Sea. The animals are well adapted to extreme climatic conditions of the area, robust constitution, longevity and resistance towards disease. So far, the studies on this breed refer to its genetics (Hirata et al., 2015), assessment of its performance influenced by feeding factors (Yordanov, 2017), reproduction (Palova and Marchev, 2009) and meat quality (Popova et al., 2015). For the development of the full performance potential of the breed, it is of crucial importance to investigate the influence of various factors affecting its health status. Some of the important parameters for its assessment are the hematological and serum biochemical profile of the animals. Palova et al. (2008) studied the hematological traits of fatteners from this breed when reared organically.The interpretation of these parameters comparing them to reference values might be difficult since most of the studies provide reference values for the modern breeds that are reared in intensive systems. Still very few report data about indigenous breeds that are reared in extensive conditions, affected by various environmental factors, such as seasonal climatic conditions. Therefore, it is necessary to complete and update the data for the profile of blood as affected by factors such as age and seasonal as well as the natural resistance in pigs from the East Balkan breed and their relationship to performance traits. Hence, this study was designed to assess the changes of the haematological and biochemical profile of East Balkan pigs depending on the age and season.

MATERIAL AND METHODS

Experimental animals, rearing and blood sampling

The trial was carried out in the Scientific Center of Agriculture – Sredets with threeage groups of East Balkan pigs – pre-weaned (n=10), growers (n=10) and sows (n=10). The growers and sows were reared according to the traditional scheme of feeding for this breed. The animals were taken daily to controlled pasture on the natural grassland (legumes 8.6%, cereals 44.7%, others 46.7%) in

Strandja mountain, as the duration of grazing depended on the climate and the pasture condition. In the evening, the animals were fed in groups with ground organic feed containing barley. The chemical composition of the grassland and barley are presented in Table 1. The pigs has *ad libitum* access to water.

Table. 1 Chemical composition of the grassland and barley

Item	Grassland	Barley
Water, %	27.31	9.05
Dry matter,%	79.69	90.95
Organic matter, %	66.93	88.59
% or Dry matter:		
Crude protein	13.48	8.09
Crude fat	2.17	0.93
Crude fiber	26.96	2.74
Minerals	7.92	2.36
Ca	1.76	0.11
P	0.62	0.45

Blood sampling was done by puncturing anterior *venacava*, the blood was then poured into test tube containing anticoagulant. Blood samples were taken in spring and summer. Cold chain was maintained during the transit of the samples from the farm to the laboratory.

Hematological and serum biochemical paramaters

Haematological analysis included the following parameters: WBC, number of Lymphocites and Granulocites, % of lymphocytes and granulocytes, RBC, HGB, HCT, mean corpuscular volume (MCV), MCH, MCHC, PLT and mean platelet volume (MPV). The paremeters were determined using automatic hematology analyser EXIGO in the Department of internal non contagious diseases in the Veterinary faculty of the Trakia University, Stara Zagora. Serum biochemical analysis was performed by automatic biochemical analyser SYNCHRON CX9 PRO and included determination of glucose, urea, TP, ALB, AST, ALT, GGT, ALP, UA, Ca, P, Mg, TG and cholesterol.

Statistical evaluation

Data were statistically evaluated by two-way ANOVA as the age, season and their interaction were included in the model. The Fit model procedure of JMP v.7 software package was used to perform the statistical analysis (JMP Version 7, SAS Institute Inc. Cary, NC). The effects were considered to be significant at $P<0.05$; $P<0.01$ and $P<0.001$. Significant differences among the means were determined using Tukey post hoc test ($P<0.05$). All data were expressed as mean values with pooled standard errors.

RESULTS AND DISCUSSION

Hematological parameters

As seen from Table 2, the age of the pigs had significant effect on the WBC as well as the number of granulocytes ($P<0.001$) and they decreased, reaching their minimum in the sows, when compared to the other two groups ($P<0.05$). This was also observed in regard of the the total platelet count ($P<0.05$). The last two parameters were also affected by the season, showing substantial decrease in their values in summer. On the other hand, the lymphocytes in the pigs remained unaffected by their age groups, however, their count and percentage increased in summer.

The increased number of leukocytes usually is associated with the presence of disease in the organism, however, it can also be attributed to strenuous exercises and feeding. Czech et al. (2017) stated that the higher WBC might occur in sows in the final stage of gestation, as well as sucking piglets. Jezek et al. (2018) reported higher WBC in younger pigs when compared to sows which is in agreement with our results. Contrary to our results, Mayengbam et al. (2014) found increase in the WBC with age, as the maximum values were observed in the adult pigs when compared to pre-weaned and growers, however, these authors detected no effect of the age on the lymphocytes which is in line with our observations.

Platelets are fragments of the cells called megakayocytes in bone marrow. Wneh stimulated by thrombopoietin, the platelets break off the megakaryocytes and enter the blood stream. Generally the low number of platelets might be associated with disease or a genetic disorder, while a higher than normal count of platelets is known as thrombocytosis and can pose serious health risks (Campbell et al., 2008). The higher PLT in this study indicated that there might be less chance of disease in pre-weaned and grower pigs in comparison to adult sows, and also in spring when compared to summer. Chu and Song (2013) reported significant decrease in PLT in fatteners in summer when compared to spring, which is in line with our results.

Table 2. Effect of the age, season and their interaction on the hematological parameters in East Balkan pigs

Treatment	WBC 11.1-22. 0 $x10^9$l	Lymp $x10^9$l	Gran $x10^9$l	Lymp% %	Gran% %	RBC 5.0-9.50 $x10^{12}$ l	HGB 99- 165 g/l	HCT, 32.0-50.0 %	MCV 51.0-68.0 fl	MHC 17.0-22.0 pg	MCHC 300-380 g/l	PLT 200- 700 $x10^9$l
Pre-weaned spring	25.35	8.47	13.42	33.03	53.23	8.76a	144.00a	46.51a	52.99	16.28	307.40	409.20
Growers spring	25.61	6.79	14.52	24.99	58.14	7.42b	127.10abc	41.13ab	55.55	17.09	308.50	484.20
Sows spring	17.97	5.64	10.23	31.22	56.97	5.97d	122.30bc	39.16abc	65.81	20.49	311.60	339.30
Pre-weaned summer	21.56	8.80	10.37	40.53	47.45	6.03cd	113.70c	33.11c	55.34	18.91	346.20	415.40
Growers summer	27.26	11.42	12.42	40.97	46.10	6.96bc	136.90ab	38.56bc	55.18	19.69	362.00	316.10
Sows summer	18.46	8.36	8.25	45.34	44.57	5.46d	127.80abc	35.11bc	64.34	23.60	373.80	222.00
Age (A)												
Pre-weaned	23.46a	8.64	11.89a	36.78	50.49	7.40a	128.90	39.81	54.17a	17.59a	326.80	412.30a
Growers	26.44a	9.11	13.47a	32.98	52.12	7.19a	132.00	39.84	55.37a	18.39a	335.25	400.15ab
Sows	18.22b	3.86	9.24b	38.28	50.77	5.72b	125.05	37.14	65.08b	22.04b	342.70	280.65b
Season (S)												
Spring	22.98	6.97	12.72	29.75	56.11	7.38	131.16	42.27	58.11	17.95	309.17	410.90
Summer	22.43	9.53	10.34	42.28	46.14	6.15	126.13	35.59	58.28	20.73	360.66	317.83
Sig.												
AxS	ns	ns	ns	ns	ns	***	***	**	ns	ns	ns	ns
A	***	ns	***	ns	ns	***	ns	ns	***	***	ns	*
S	ns	**	**	***	***	***	ns	***	ns	***	***	*
Pooled SEM	0.83	0.45	0.45	1.31	1.11	0.17	2.18	0.88	1.01	0.37	5.65	22.43

P<0.05; **P<0.01; ***P<0.001. Means connected with different letters are statistically different (P<0.05).

In regard to the RBC, both age and season as factors affecting this trait, interacted significantly ($P<0.001$). It was reflected by the different patterns that changes of the parameter followed with age, namely the gradual decrease in its values from the pre-weaned to growers and sows in spring, however in summer, the pre-weaned pigs showed lower RBC when compared to the growers ($P<0.05$). This was also observed in regard to the HGB and HCT. Seasonal variations were found only in RBC and HCT in pre-weaned pigs showing lower values in summer in comparison to spring season. Contrary to our results, Mayengbam et al. (2014) found increase in the RBC and HCT in the adult sows, when compared to pre-weaned and growers of and indigenous breed of pigs, however the same authors in another study with the same breed (Mayengbam et al., 2017) showed seasonal variations in these traits in growers and adult sows. MCV and MCH were significantly affected by the age of the animals ($P<0.001$), showing increase in the sows when compared to the pre-weaned and growers ($P<0.05$). Furthermore, MCH differed between seasons, showing higher content in summer, which coincided with the higher values of MCHC observed in this season. Mayengbam et al. (2017) reported effect of the age on the MCV and MHC showing decrease of these parameters between the pre-weaned and grower stage, and then increase in the adult sows. In our study we observed higher values of both MCV and MHC in the sows when compared to both pre-weaned and growers. Eze et al. (2010) did not report any difference in these parameters in piglets and adults. Furthermore, in the study of MCV decreased, while MHC and MCHC increased from winter to summer. This partly coincides with our results showing higher values of MHC and MHCH in summer, when compared to spring. The lower values of these parameters as well as the changes in the HB and HCT, especially in pre-weaned pigs might indicate inflammatory response (Odink et al., 1990).

Serum biochemical parameters

Important interactions of age and season were found in regard to the most serum biochemical parameters that we use to describe the health status of the East Balkan pigs in this study (Table 3). Both factors interacted in creatinine, total protein and albumin ($P<0.001$). Although generally, creatinine increased with the age and lowered in summer, these changes followed different trend. In spring the concentration remained lower in pre-weaned pigs compared to growers and sows ($P<0.05$), however in summer the difference between the pre-weaned and growers were less pronounced. Seasonal changes existed only in pre-weaned and growers, while in sows the values remained relatively constant. Total protein increased gradually between all the age groups, while significant increase in the albumin existed only in the adult sows. These changes were observed only in summer, however significant difference between seasons were found. The increase in the total protein with age was observed in other species such as ruminants (Zarghan, 1994; Ahmadi et al., 2014; Habibu et al., 2017). This might be attributed to the higher albumin levels observed in the adult animals.

Table 3. Effect of the age, season and their interaction on the serum biochemical parameters in East Balkan pigs

Treatment	Glucose mmol/l	Urea mmol/l	Creatinine µmol/	TP g/l	ALB g/l	AST U/l	ALT U/l	GGT U/l	ALP U/l	UA µmol/	TG mmol/l	Cholesterol mmol/l
Pre-weaned spring	8.65	7.87	97.10b	74.90b	44.90a	129.80a	80.10a	50.10	472.90	115.70a	1.27ab	4.02a
Growers spring	9.57	6.09	135.20a	78.21ab	41.64ab	103.50a	54.40b	33.50	317.20	76.80ab	0.93b	3.38b
Sows spring	5.65	5.27	130.30a	78.64ab	43.31ab	92.80ab	51.40b	40.00	82.70	33.60b	0.54c	2.30c
Pre-weaned summer	5.39	5.23	73.40b	63.98c	31.10c	35.70c	51.90b	85.70	357.90	52.30b	1.27ab	3.13b
Growers summer	7.42	4.60	93.40b	71.63bc	33.52c	39.60bc	45.30b	60.80	217.30	46.60b	1.38a	2.83bc
Sows summer	4.69	4.31	139.70a	83.28a	40.57b	86.20abc	67.20ab	66.80	62.30	51.40b	0.93b	2.44c
Age (A)												
Pre-weaned	7.02a	6.55a	85.25a	69.44a	38.00a	82.75	66.00a	67.90a	415.40a	84.00a	1.27a	3.57a
Growers	8.50a	5.35b	114.30b	74.92b	37.58a	71.55	49.85b	47.15b	267.25b	61.70ab	1.16a	3.10b
Sows	5.17b	4.79b	135.00c	80.96c	41.94b	89.50	59.30ab	53.40ab	72.50c	42.50b	0.73b	2.37c
Season (S)												
Spring	7.96	6.41	120.87	77.25	43.28	108.70	61.97	41.20	290.93	75.35	0.91	3.23
Summer	5.84	4.71	102.17	72.96	35.06	53.83	54.80	71.00	212.50	50.10	1.20	2.80
Sig.												
AxS	ns	ns	***	***	***	**	***	ns	ns	**	*	**
A	***	***	***	***	***	ns	*	*	*	**	***	***
S	***	***	***	***	***	***	ns	***	***	**	***	***
Pooled SEM	0.37	0.23	4.05	1.11	0.75	6.68	2.73	3.75	27.64	5.78	0.05	0.09

*P<0.05; **P<0.01; ***P<0.001. Means connected with different letters are statistically different (P<0.05).

Higher uptake and utilization of dietary protein for growth and development in young animals as compared with adults may be responsible for the low blood total protein. Significant effect of the season ($P<0.001$) and of age ($P<0.05$) was found in regard of AST and ALT respectively, though these factors significantly interacted. ALT and AST are two of the most reliable markers of hepatocellular injury or necrosis.Surprisingly, in summer we observed
dramatic decline in the AST, which was well defined in the pre-weaned and growers. Increase of the ambient temperature is associated with higher levels of increases which was showed in the studies of Nazifi et al. (2003) in goats and Chmielowiec-Korzeniowska et al. (2012), which we failed to observe.

Furthermore, important interactions were observed in regard to the UA content ($P<0.01$), Mg ($P<0.001$), serum TG ($P<0.05$) and cholesterol ($P<0.01$). All these parameters showed considerable decrease with age, and reaching minimal values in sows, however depending on the season.

Also differences between seasons were also detected but not in all age groups and not following the same patterns, which confirmed the significant interaction between both studied factors. The changes in the Mg content that we observed in this study does not agree with the findings of Mayengbam et al. (2017), while on the other hand it corresponds to the changes in the contents of Ca. The contents of TG and cholesterol decreased with age which corresponded with the reported effect of age in other studies (Yeom et al., 2012). Also we observed dependence of these parameters with the season showing decrease in the summer for the cholesterol, which could be associated with the changes in the diet of the animals.

Regardless of each other age and season affected the concentration of glucose and urea ($P<0.001$), showing decrease with age and lower values in summer. Contrary to us, Chmielowiec-Korzeniowska et al. (2012) found significant increase in the glucose levels in fatteners at higher temperatures (when comparing winter and summer). Also Chu and Song (2013), reported higher urea in pigs when comparing summer to spring which contradicts our results. On the other hand, Hooda and Upadhyay, 2014) reported decreased glucose with increasing temperature in kids and Yeom et al. (2012) showed lower urea with age in pigs which is in line with our findings. The age related changes in these two parameters might be considered normal, since their values do not deviate from the reference intervals determined by Friendship et al. (1984).

The same age variations were observed in regard to the GGT and ALP ($P<0.05$) which lowered their content with increasing the age of the groups, however, they did not showed the same affect of the season. Generally, GGT increased in summer, while ALP showed higher concentrations in spring. Age affected the content of Ca ($P<0.05$) and P ($P<0.001$) as it decreased in the older animals (Table 4). Significant decrease was also found in the summer in regard of these parameters. Our observations coincided with Chmielowiec-Korzeniowska et al. (2012), that showed higher GGT of fatteners in summer. Furthermore, our results are in line with those reported by Mayengbam et al. (2014), reporting decrease in ALP in adult sows and also lower values in the summer. The decrease in the ALP

might be associated with lower demands for this enzyme for skeletal growth that is observed in older animals (Rosol and Capens, 1999).

Table 4. Effect of the age, season and their interaction on the mineral contents of serum in East Balkan pigs

Treatment	Ca mmol/ml	P mmol/l	Mg mmol/l
Pre-weaned spring	4.19	3.29	1.50a
Growers spring	3.66	2.75	1.61a
Sows spring	3.76	2.06	0.96b
Pre-weaned summer	1.98	2.51	0.86b
Growers summer	1.50	2.15	0.84b
Sows summer	1.60	2.03	0.87b
Age (A)			
Pre-weaned	3.09a	2.90a	1.18a
Growers	2.58b	2.05b	1.22a
Sows	2.68ab	2.45b	0.91b
Season (S)			
Spring	3.87	2.70	1.36
Summer	1.69	2.23	0.85
Sig.			
AxS	ns	ns	***
A	*	***	***
S	***	**	***
Pooled SEM	0.16	0.09	0.05

*P<0.05; **P<0.01; ***P<0.001. Means connected with different letters are statistically different (P<0.05).\

Furthermore, as stated by Antonov and Malchevski (1983), up to 6 months, the alkaline phosphatase in pigs is of bone origin, while in adult sows it it from liver. This corresponded with the lower levels of P and Ca in the serum with increasing of the age of the pigs. On the other hand, the APL decreased in summer, which can be associated with the higher temperatures.Hooda and Singh (2010) and Sejan et al. (2010) found significant reduction in ALP in respectively in buffalo heifers and goats, which in the older animals can be attributed to disfunction of liver at high ambient temperature.

CONCLUSIONS

In conclusion, the age and season, as well as their interaction affected differently the hematological and serum biochemical parameters in East Balkan pigs. Their changes described the adaptive capabilities of the different age groups during two different season, however, more experiments are needed to fully clarify the

dependencies of the blood and serum parameters of the various factors which will allow to use them to improve the performance traits in East Balkan breed.

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- ACKNOWLEDGMENTS

If received significant help in designing, or carrying out the work, or received materials from someone who did a favour by supplying them, their assistance must be acknowledged. Acknowledgments are always brief and never flowery.

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