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The Peculiarities of Carbon Metabolism in the Ears of C3 Cereals: The Carbon Metabolism and Key Genes Expression in The Photosyn-thetic Active Components of the Ear of Cereals

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Abstract

The ear of C_3 cereals makes an important contribution to yield formation, but the mechanisms ensuring this phenomenon are not completely elucidated. In this article was performed a comparative study of several key components that characterize the C_3 and C_4 carbon metabolism in the ear, flag leaf of cereals and in the tassel, leaf of maize plant. In the photosynthetic active components of the ear of cereals were registered higher activity of PEPC, compared to the flag leaf. However, isotopes discrimination did not show a difference between the ear and the flag leaf. The content of metabolites associated with photorespiration demonstrated higher Serine levels in the ear compared to the leaf. This peculiarity, on the background of high expression levels of RbcS and low expression of PEPC, assisted by relative high levels of Glycolate, Glycine and Glycerite, may indicate the existence of more active photorespiration cycle in the ear or the presence of glycine a pump, characteristic for the plants with C_3 - C_4 intermediate metabolism. Also, in the ear components was registered lower expression of GOX, compared to the flag leaf. The obtained results demonstrate a specific metabolism in the ear components of cereals, placing the ear in an intermediate zone, similar to C_3 - C_4 plants.

Keywords: Carbon Isotopes Discrimination; Carbon Metabolism; Cereals Ear; Enzymes Activity; Gene Expression; Photorespiration; C_3 Plants; C_4 Plants.

Abbreviations:

Chl	:	Chlorophyll
GC - MS	:	Gas Chromatography-Mass Spectrometry
GDC - H	:	Glycine Decarboxylase Subunit H
Gly	:	Glycine
GOX	:	Glycolate Oxidase
MDH	:	Malate Dehydrogenase
NA	:	Not available
NAD - ME	:	Malate Decarboxylase, NAD Dependent Malic Enzyme
NADP - ME	:	Malate Decarboxylase, NADP Dependent Malic Enzyme

PEPC	:	Phosphoenolpyruvate Carboxylase
PPDK	:	Pyruvate Orthophosphate Di Kinase
RbcL	:	Rubisco Large Subunit
RbcS	:	Rubisco Small Subunit
Rubisco carboxylase/oxygenase	:	Ribulose-1,5-bisphosphate
Ser	:	Serine

Introduction

Discovering the phenomenon of the lack of apparent photorespiration in the reproductive organs of the C_3 cereals [1] revealed also the fact that in cereals only the leaf registers C_3 photosynthesis. The rest of organs (ear, stem, leaf sheath, peduncle) have a CO_2 exchange kinetics similar to the maize C_4 leaf, also lacking apparent photorespiration [2]. These results suggested the assumption that cereal plants productivity is ensured by two mechanisms of carbon assimilation. One is of C_3 type, localized in the in the leaves and the second one localized in the ear photosynthetic active components, that has some elements of the C_4 photosynthesis that limits the CO_2 losses in post-illumination phase [3].

From another part it is known that majority of enzymes necessary for C_4 pathway are already present in C_3 plants, but in lower quantities and it is supposed that C_3 plants are preconditioned for C_4 metabolism [4,5]. In the last decade appeared many works that demonstrate the C_4 preconditioning assumption of the C_3 plants.

In the cells surrounding vascular bundles in tobacco the radioactive signal from labeled malate was found in soluble sugars and amino acids, suggesting that CO_2 originates from photosynthetic malate. It is considered that the genes involved in C_4 photosynthesis were recruited from orthologs present in C_3 species through modification of their spatial expression pattern and restriction to one cell type [6].

One of the main differences between C_3 and C_4 plants lies within the content and activity of PEPC. This is a specific C_4 enzyme, active in cytosol of mesophyll cells, that fix CO_2 in organic acids with four atoms of carbon: malate or aspartate. Nevertheless in the last decade several research teams reported about the presence and considerable activity of PEPC in C_3 plants: in the cells surrounding vascular bundles in tobacco [4] and *Arabidopsis* [6], in the ear of *Tr. aestivum L.* [7], assuming its role in CO_2 fixation. Plants with C_4 photosynthesis have much more PEPC than Rubisco and need much less Rubisco in order to fix the same quantity of CO_2 compared with C_3 plants. This results in a greater efficiency of N utilization [8,9].

By mRNA-Seq 5 different species from Flaveria genus were compared (C_3, C_4 and C_3-C_4 types of photosynthesis) in order to quantify the transcriptome differences in the leaf. In the C_4 Flaveria plants the biggest number of genes with reduced expression belonged to photorespiration group. Surprisingly was the fact that in C_3-C_4 intermediate species of Flaveria did not registered intermediate characteristics of genes expression levels: the expression level of photorespiration associated genes was even higher than in C_3 species of Flaveria, but the expression levels of Calvin-Benson cycle genes was C_3 similar [10]. Despite the fact that these species assimilate up to 50% of the carbon through C_4 pathway.

From literature data it is known that C_3 - C_4 intermediate species of *Flaveria* do not register intermediate characteristics for photosynthesis and photorespiration genes expression compared to C_3 and C_4 species. Moreover, the abundance of the majority of the transcripts associated with the photosynthesis and the photorespiration were greater than in the C_3 species, the expression of the majority of the genes of Calvin-Benson was C_3 like with exception of RbcL, the expression level of which was significantly lower than in the C_3 species [10].

Taking into account all mentioned above in this article was performed a comparative study of several key components that characterize the C_3 and C_4 metabolism: the PEPC activity; the relative contents of PEPC, RbcL and GDC-H; carbon isotopes discrimination; profiling of the main metabolites associated with photorespiration and the relative expression of RbcS, PEPC, GDC-H, GOX genes. All these investigations were performed for the flag leaf and ear components (glume, lemma and awn) in comparison to the maize leaf and tassel, in the same growing conditions and developmental stages.

Materials and Methods

Study Objects and Plant Growth Conditions

C_3 plants of *Tr. durum* L. (variety Hederiform 335), *Triticale* (variety Ingen 93) and *Zea mays* (line 459 and hybrid RF7xW47) served as study objects. All these genotypes were grown in the experimental fields of the Institute of Genetics, Physiology and Plants Protection of the Academy of Science of Republic of Moldova and in the greenhouse of the Umea Plant Science Center (Sweden). Greenhouse conditions were: 20°C/15°C - day/night temperatures, 50%-70% relative humidity, 16h photoperiod with light intensity of 700 µmol/m2/s. Cereal plants were grown in pots of 3 liter, in two rows (3cm between rows) and fertilized with Rika-S (N-P-K, 7-1-8) through an automated irrigation system. At the stage of 2-3 leaves, plants were vernalized during one month:

15°C/5°C day/night temperatures, 50%-70% relative humidity and 8h photoperiod. After verbalization plants were returned back to the greenhouse. Maize plants (hybrid W47xRf7) were grown at the same greenhouse conditions, but in 10-liter pots. Biological material from maize (leaf and tassel glumes) were collected at the beginning of tassel flowering. In case of *Tr. durum* and *Triticale* biological samples were collected at two developmental stages (earning and milk/waxy (ripening) from four different tissues: flag leaf, awn, lemma and glume). All samples were collected in three biological repetitions and each repetition was composed by pooled material from three individual plants grown in different pots. Samples collection started after 6-8h of illumination, when photosynthesis reaches its maximum capacity. Samples were ground manually in liquid nitrogen, using a pestle and mortar, until a fine powder was obtained and stored in freezer at -80°C. The same stock of ground material was used for all the downstream experiments. The field grown plants were used for gas exchange analysis, anatomical structure and chloroplast ultrastructure determination. Plants from the greenhouse were used for enzyme activity assays, western blots, gene expression analysis and metabolite profiling.

Chlorophyll CONTENT, PEPC Activity and Western Blot

Chlorophyll quantification was done using UV/VIS Lambda 18 Perkin-Elmer spectrophotometer at the three wavelengths: 646.6nm, 663.6nm, 750nm. All calculations were done according to [11]. Total proteins extraction and PEPC activity was done according to [12], monitoring photometrically (at 340nm) the oxidation of NADH through coupling the carboxylase reaction with MDH, at optimal temperature of 30°C, using HALO DB-30 UV-VIS (Dynamical) spectrophotometer. Protein extraction buffer contained 50mM HEPES-NaOH pH8, 10mM MgCl2, 5mM DTT, 1mM EDTA, 20% Glycerin, 1mM Pefabloc SC (Roche), 2% PVP. Reaction buffer contained 50mM HEPES-NaOH pH8, 5mM MgCl2, 5mM Glucose 6-phosphate, 1mM NaHCO3, 20% Glycerin, 0.2mM NADH, 6U/ml Malatdegydrogenase (MDH), pH8. In order to start the reaction to 850µl of reaction buffer was added 100µl protein extract and 50µl (2.5mM) PEP for positive reactions or water for controls. The activity of PEPC was calculated based on reaction rates recorded within a range where the increase in A340nm absorbance was linear. Protein concentrations were measured according to Bradford method using Bio-Rad kit in microplate assay and Spectra MAX 190 spectrophotometer. All isolated proteins were stored at -80ºC.

For Western blot the proteins were isolated from the same grinded material according to [13]. Protein extracts from different photosynthetic active components were resolved through SDS-PAGE [14] with different concentrations of acrylamide in order to ensure good protein separation. After electrophoresis proteins were electro-transferred on the nitrocellulose membrane [15]. For immunodetection polyclonal antibodies (Agrisera) were used in different dilutions: RbcL (1:5000), PEPC (1:500) si GDC-H (1:500). Coupled antibodies on membranes were detected through chemiluminescence using Amersham ECL Western blotting detection reagents and Fujifilm LAS-3000 Imager, according to manufacturer's recommendations.

Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from 20mg of grinded material using RiboZol (Ameresco) reagent, according to manufacturer's recommendations, in three biological repetitions. Isolated RNA concentration was checked at Nanodrop 2000 spectrophotometer and degradation level through gel electrophoresis (2% agarose, stained with Gel Red or Midori Green). After DNase treatment (DNA-free kit, Ambien) and RNA concentration re-measurement 1µg of total RNA was revers-transcribed using qScript cDNA Synthesis kit (Quanta), according to manufacturer's recommendations. Groups of five cDNA samples were pooled and used for negative RT reactions (reverse transcription without RT enzyme) - controls for DNA contamination in qPCR reactions. Isolated RNA samples were stored at -80°C and cDNA samples at -20°C.

qPCR Analysis

Samples processing and qPCR analysis were done according to MIQE guidelines [16]. Using NCBI database coding sequences of PEPC and Rubisco (small subunit) genes for *Zea mays, Triticum* sp. and coding sequences for GOX, GDC-H only for *Triticum sp.,* (Table 1) were identified.

Gene	Primers sequence, 5'.....3'	Amplicon size, bp	PCR efficiency
Triticum RbcS mRNA, (2 Exons)	CGC GTC AGC AAT GGC GGA AG	143	*Tr.durum/Triticale* 1.85/1.82
	GTC GAC CTG CTT CAG GAG GG		
Triticum PEPC mRNA (2 Exons)	GGA GAC CCA GAA GCT GCT TC	213	2.00/2.07
	GAT CCT CTT CAA TGT GTA TGC CTG		
Maize Rubisco small subunit (RbcS)	GGTGTACAAGGAGCTGCAGGAGGCCAT	173	1.85
	GGCAGAGGCATGGCCATGGGTCG		
Maize PEPC	AGAACTCAAGCCCTTTGGGAAGC	238	1.82
	GTCGGCGAACTCCTTGGACAGC		
Triticum GDC-H	CACGAGTGGGTCAAGAACGA	155	1.88/1.92
	GCCTTCACACTCTCCACGTT		
Triticum GOX1	TGTATCGGAGTACCAGGCCA	120	1.89/1.85
	ATCCTGGAGAATGCCTCCCT		

Table 1: The sequence of used primers, amplicon size and reaction efficiencies for the detection of RbcS, PEPC, GDC-H and GOX1.

Primers where constructed using Primer3 software and synthesized commercially (Eurofins). The same primers were used for the analysis of *Tr. durum* and *Triticale* samples. The sequences of several potential reference genes were picked from the articles - 5 genes for maize and 3 for *Tr. durum* (Table 2), *Triticale* [17,18]. The experimental design was based on three biological repetitions and each biological repetition was processed in three technical repetitions. Amplification conditions were tested on a dilutions series of cDNA samples pool. PCR products were analyzed through 2% agarose gel electrophoresis in order to verify if only one fragment of expected size was amplified - crucial moments in qPCR.

For amplification we used EVA-Green kit (qPCR Eva Green qPCR Master mix (ABM)), half reactions and two step programs (40 de cycles: 94°C - 60sec, 60°C - 60sec) followed by melting curve analysis. Total volume of one reaction was 10µl and contained 1x EVA-Green reaction buffer, 10µM of each primer and 2µl of diluted cDNA (1:20). For standard curve we used a dilution series of pooled cDNA from each sample of the same genotype. All amplifications were run on Bio-Rad CFX70 machine and CFX Manager Software was used for data extraction. Obtained data were analyzed using the same method - determination of normalized relative expression level according to the formula:

$RQ=(E_{ref}\text{^}Cq_{ref})/(E_{target}\text{^}Cq_{target})$, [19]

Where:

E - efficiency of amplification reactions for reference and target genes;

Cq - cycle of quantification for reference and target genes.

Obtained relative quantities were log transformed in order to have them normal distributed and analyzed statistically using Excel.

Gene	Gene annotation/ gene product	Primers sequence, 5'.....3'	Amplicon size, bp	qPCR efficiency calculated from slope
Tr. durum/*Triticale*				
Ta2291	ADP-ribosylation factor	GCTCTCCAACAACATTGCCAAC GCTTCTGCCTGTCACATACGC	165	1.99

Ta54227	Cell div. control prot. (AAA-superfam. ATPases)	CGATTCAGAGCAGCGTATTGTTG AGTTGGTCGGGTCTCTTCTAAATG	227	NA
Ta2776	Similar to RNase L inhibitor-like protein	CAAATACGCCATCAGGGAGAACATC CGCTGCCGAAACCACGAGAC	242	1.94
Zea mays				
CUL	Cullin	CAGGTGGGGTATTCTTGGTG ATGTTCGGGTGGAAAACCTT	274	NA
FPGS	Folylpolyglutamate synthase	ATCTCGTTGGGGATGTCTTG AGCACCGTTCAAATGTCTCC	132	1.86
UBCP	Ubiquitin carrier protein	TCCAGTGCTACAGGGAAGGT GTTAGTTCTTGAGCCCACGC	231	NA
MEP	Membrane protein PB1A10.07c	GAAGAGCCGCAAAGTTATGG ATGGTAGAAGTGGACGCACC	203	1.97
LUG	Leunig	TGTACTCGGCAATGCTCTTG TTTGATGCTCCAGGCTTACC	178	NA

Table 2: Primers sequences for reference genes, amplicon size and reaction efficiencies.

Reference Genes Screening

To screen potential reference genes Norm Finder software was used. Due to the little number of samples in case of maize samples, the reference gene was selected with CFX manager software during qPCR data analysis. The best gene combination was MEP and FPGS (Table 2). In case of *Tr. durum* and Triticale the genes with lowest expression stability levels (M) having respectively the most stable expression levels (Table 3) were selected.

Reference gene	Expression stability level (M)	
	Tr. durum	*Triticale*
Ta2291	0,136	0,267
Ta54227	0,215	0,264
Ta2776	0,211	0,174

Table 3: Expression stability levels of candidate reference genes for maize, *Tr. durum* and *Triticale*.

Metabolites Analysis with GC-MS

Metabolites analysis was performed on the Metabolomics Platform at the Umea Plant Science Center (Sweden). Samples for metabolomics were prepared and processed according to [20] and for data analysis SIMCA software was used.

Total Carbon Isotopes Discrimination

For this analysis only the samples of maize leaf and Triticale flag leaf, ear components were selected. Approximately 1mg of grinded material was dried out at 70°C for 18h and after that the relative abundance of ^{13}C and ^{12}C was determined using Isotope ratio mass spectrometer (Delta, Thermo Fisher Scientific) and Elemental analyzer (Flash EA 2000, Thermo Fisher Scientific).

Results and Discussion

As mentioned previously the necessity to update research and discussions in the field of CO_2 assimilation mechanisms and carbon metabolism in the ears of C_3 cereals appeared as a consequence of the last results regarding the CO_2 exchange in the ear [3]. Our results (CO_2 exchange kinetics and compensation point, anatomical structure and chloroplasts ultrastructure in photosynthetic active components of the ear) demonstrated eloquently that in the ear of cereals (*Tr. durum* and *Triticale*) are present functional and structural elements of C_3 and C_4 types of photosynthesis. Based on these results were studied several key components of the carbon metabolism in the ear of cereals: the content and activity of key photosynthetic enzymes (Rubisco, PEPC, GDG-H); expression level of RbcL, PEPC, GDC-H and GOX; contend of the metabolites associated with photorespiration (glycolate, glycerite, serine and glycine).

PEPC Activity in Photosynthetic Active Components of the Ear

One of the main differences between C_3 and C_4 plants lies within the content and activity of PEPC. This is a specific C_4 enzyme, active in cytosol of mesophyll cells, that fix CO_2 in organic acids with four atoms of carbon: malate or aspartate. Nevertheless in the last decade several research teams reported about the presence and

considerable activity of PEPC in C_3 plants: in the cells surrounding vascular bundles in tobacco [4] and *Arabidopsis* [6], in the ear of *Tr. aestivum L.* [7], assuming its role in CO_2 fixation. Taking this into account the PEPC activity was determined in the flag leaf and ear components of C_4 (maize) and C_3 (*Tr. durum*, Triticale) plants. Obtained data were normalized against total chlorophyll content for each sample (Figure 1). PEPC activity in *Tr. durum* and *Triticale* was determined for earning phase and ripening phase, but for maize samples - beginning of tassel flowering. Recorded PEPC activity for maize (C_4), *Tr. durum* and Triticale (C_3) corresponds to data known in the literature [12]: PEPC activity in C_4 plant is 10-20 times higher than in C_3. For cereals ear components obtained values were greater than those for the flag leaf, with exception of awn, earning phase, were PEPC activity was similar to the flag leaf (Figure 2). In general, for earning phase, obtained values were greater compared to ripening phase. Maximum levels of PEPC activity was registered for Triticale lemma and for the glume, 2,04 and 1,4µmol/min/mg Chl respectively.

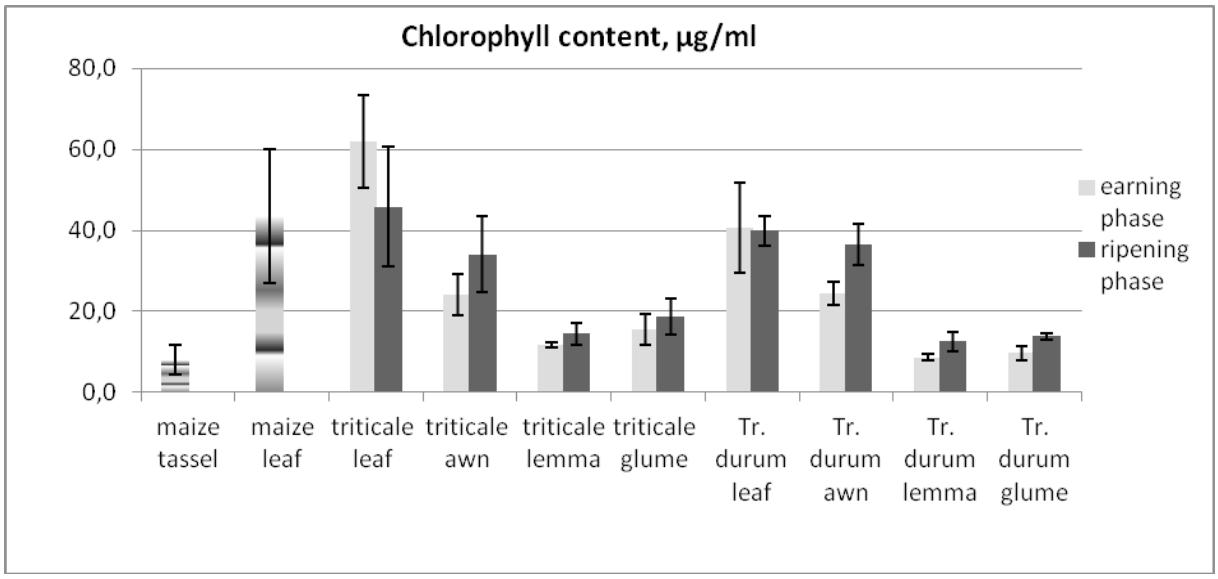


Figure 1: Chlorophyll content in maize (C_4) and *Tr. durum*, *Triticale* (C_3) samples harvested at the two developmental stages. The average of 3 biological repetitions ± 95% confidence interval is presented.

Similar results were obtained for *Tr. Aestivum L.*, where for dough-development stage of the awn was registered PEPC activities (1.7 µmol mg-1 protein min-1) double higher than in the flag leaf [7]. Taking into account the high demand in carbohydrates and proteins for grain formation the authors concluded that PEPC in the ear is actively involved in the CO_2 fixation in the light and CO_2 respired in the dark or light. The presence of awns plays an important role in CO_2 assimilation because they increase considerable the photosynthetic active area of the ear. Also the high thermo-tolerance and drought resistance of the ear is explained through more abundant presence and activity of PEPC comparing to the flag leaf [21]. Despite the suggestions of other authors, we registered higher PEPC activity in the ear components comparing to the flag leaf.

Western blot of PEPC, RbcL and GDC-H Proteins

In order to estimate the relative amount of some key enzymes in photosynthesis and photorespiration Western blot was performed using polyclonal antibodies (Agrisera, SE). It is known that Rubisco content in C_3 plants is much higher than in C_4 plants [9]. Our hypothesis was that in the ear components, lacking apparent photorespiration, there should exist a mechanism of CO_2 re-fixation that may result in lower amount of Rubisco. Obtained data partially confirmed that hypothesis. In *Tr. durum* and Triticale samples the most abundant protein was RbcL in both developmental stages, as expected for a C_3 plant (Figure 3). Nevertheless, for the flag leaf the amount of detected protein was much higher compared with the ear components. For the maize leaf (C_4) this protein was less abundant comparing to the rest of samples.

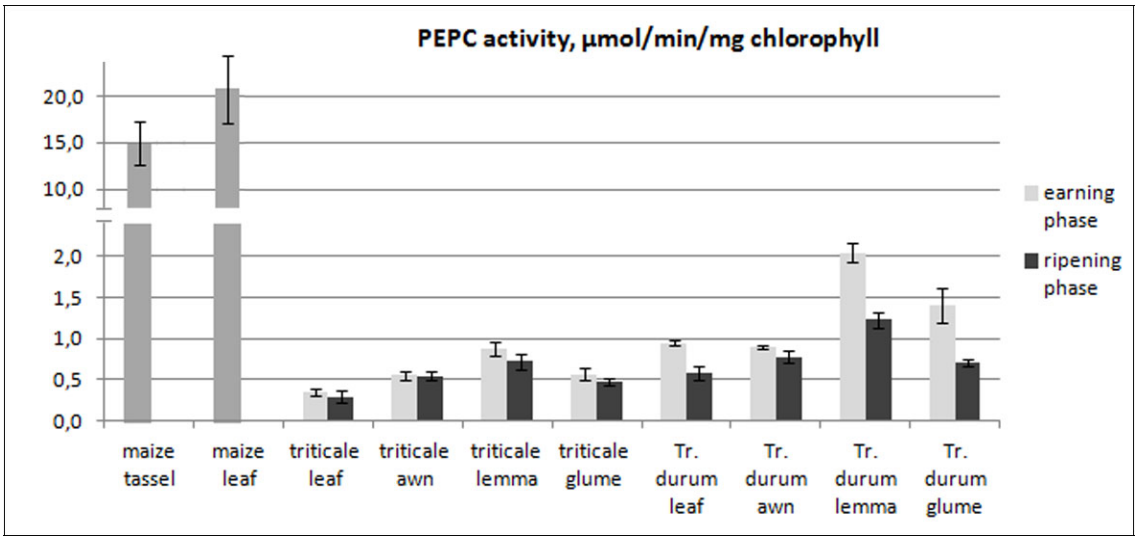


Figure 2: Activity of PEPC in *Tr. durum,* Triticale at two developmental stages and maize samples. The average of 3 biological repetitions ± 95% confidence interval is presented.

Next detected protein was PEPC and its content in maize leaf was very abundant compared with RbcL, as expected for the C_4 plant. In case of *Tr. durum* and Triticale lower quantities of PEPC were detected, besides the fact that on the gel 100 µg total protein was loaded. The greatest band intensities for both types of cereals were obtained for the flag leaf and awn (Figure 3 A, B). One possible explanation may be due to the usage of the polyclonal antibody for maize PEPC, on the other hand it is possible to say that this protein is present and active in the ear components of C_3 cereals, especially in the awn. This fact confirms that PEPC enzyme with potential carboxylation function is present in C_3 plants, but in much lower concentration compared to C_4 plants (Figure 2 A, B). The last protein that we detected was H protein of glycine-decarboxylase complex (GDC-H). Protein H plays a crucial role for the activity of entire GDC complex in mitochondria [22] and may be used as an indirect indicator of photorespiration.

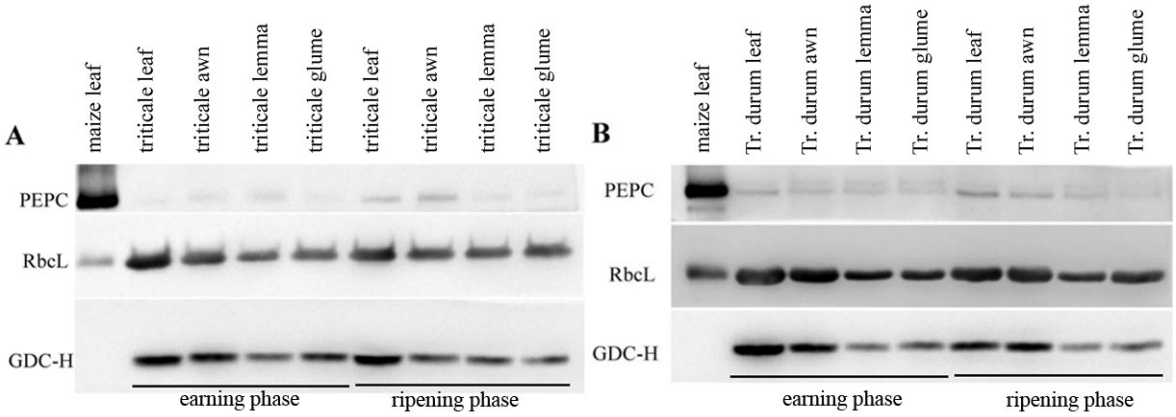


Figure 3: Western-blot analysis of PEPC, RbcL and GDC-H proteins in maize, Triticale (A) and *Tr. durum* (B) samples. In case of the RbcL detection, for maize samples, 30µg total protein was loaded and for cereal samples - 4 µg; in case of PEPC for maize samples 30µg total protein was loaded and for cereal samples - 100 µg; in case of GDC-H detection for all samples 30µg total protein was loaded. The gels were resolved on SDS-PAGE (8% for RbcL, PEPC and 18% for GDC-H), transferred on nitrocellulose membrane and probed with polyclonal antibodies. The membranes were developed using ECL standard reagent.

Was loaded 30µg of total protein on the gel, however nothing was detected in case of maize leaf (Figure 3 A). For cereal samples the maximum amount was for the flag leaf and awn samples, with lower amounts in the rest of ear components for both developmental stages (Figure 3 A B).

Carbon Isotope Discrimination in Triticale Ear Components

Carbon isotopes $^{13}CO_2$ and $^{12}CO_2$ diffuse differentially through the leaf and because of Rubisco preferences for $^{12}CO_2$ plants with different type of carbon metabolism (C_3, C_4, C_3-C_4 and CAM) have a specific signature after isotopes discrimination [23]. In order to determine the specificity of CO_2 fixation metabolism in ear components total carbon isotopes discrimination was performed. We selected maize leaf as a C_4 control and Triticale flag leaf as a C_3 control. Clear differences were obtained for isotopes signature in maize leaf, that had less negative values compared to Triticale samples. For Triticale no difference was between the flag leaf and ear components (Table 4). Similar results were obtained for *Flaveria* species with intermediate type of metabolism (C_3 - C_4), where no difference was registered in carbon isotopes discrimination compared with C_3 flag leaf of *Flaveria* [10], despite the fact that intermediate *Flaveria* species fix up to 50% of carbon through C_4 pathway. Theoretically, lack of C_4 signature in the ear components does not necessarily mean complete absence of C_4 metabolism in respective photosynthetic tissues, taking into account also higher PEPC activity compared with the leaf (Figure 2).

Relative expression level of some photosynthetic and photorepiratoric genes (RbcS, PEPC, GDC-H, GOX)

The majority of enzymes necessary for C_4 pathway are already present in C_3 plants, but in lower quantities and it is supposed that C_3 plants are preconditioned for C_4 metabolism [3]. In the cells surrounding vascular bundles in tobacco the radioactive signal from labeled malate was found in soluble sugars and amino acids, suggesting that CO_2 originates from photosynthetic malate [6].

		Atom%
	d ^{13}C [‰]	F_C / %
sample	0	1,111233
maize leaf	-13,20	1,096730
Triticale flag leaf	-32,81	1,075162
Triticale awn	-31,34	1,076779
Triticale lemma	-32,58	1,075419
Triticale glume	-32,50	1,075502

Table 4: Carbon isotope ratios in dried material of maize ant *Triticale* samples.

It is considered that the genes involved in C_4 photosynthesis were recruited from orthologs present in C_3 species through modification of their spatial expression pattern and restriction to one cell type [5]. In order to detect differences and similarities in CO_2 assimilation metabolism between ear components of C_3 cereals and maize leaf (C_4) we determined the relative expression levels of several key genes in C_3 and C_4 photosynthesis (Table 1). Relative expression data were normalized against a reference gene, individually detected for maize and cereals, using several candidate genes and Norm Finder software (Table 2, 3).

In case of maize leaf and tassel the expression level of PEPC and RbcS was determined. In the leaf PEPC expression was 5.6-fold higher than RbcS (Figure 4). Plants with C_4 photosynthesis have much more PEPC than Rubisco and need much less Rubisco in order to fix the same quantity of CO_2 compared with C_3 plants. This results in a greater efficiency of N utilization [8,9]. In maize tassel the expression level of both genes was approximately at same level, but much lower compared with the leaf. For example, PEPC expression in the leaf was 4.9-fold higher than in the tassel. Although glumes of maize tassel are photosynthetic active and their chlorophyll content is very low (Figure 1). Relative gene expression data correspond to Western blot data for these proteins in maize, although in case of the proteins RbcL instead of RbcS was determined (Figure 3).

For *Tr. durum* and *Triticale,* the relative expression of four genes was analyzed: RbcS, PEPC, GDC-H and GOX (Table 1). We chose these genes in order to characterize photosynthesis and the activity of photorespiratory cycle in the flag leaf and ear components.

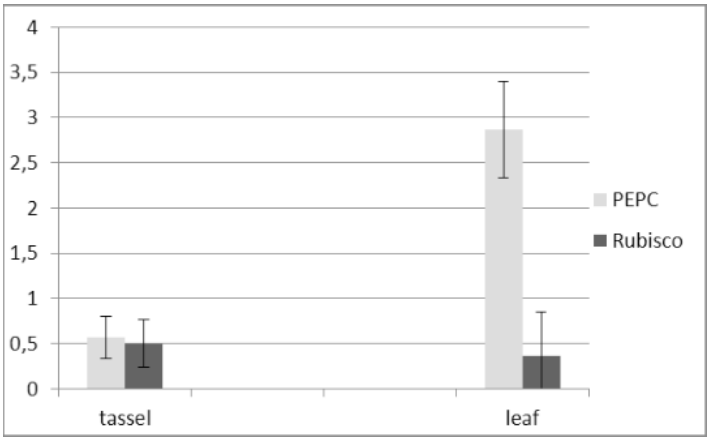


Figure 4: Normalized relative expression level of PEPC and Rubisco genes in maize samples (tassel and leaf) collected at the tassel flowering stage, in log2 scale. The average of 3 biological repetitions ± 95% confidence interval is presented.

For the ear components we obtained specific expression profiles for these genes. According to the data known in the literature the ear of *Tr. aestivum* has anatomical, ultrastructure and physiological advantages for CO_2 fixation compared with the flag leaf. These advantages are more evident especially at the ripening stages. Increased stomata number and PEPC activity demonstrates that the ear has a superior capacity to adapt to different ecological conditions [24]. From another part, different ear components have a specific role in photosynthesis or protection of the grain. Microarray analysis of oat ear components revealed that lemma and paleo express more defense related genes, but in the awn more genes associated with photosynthesis are expressed [25]. These data suggest that lemma and paleo have defense/protection functions, but awn has photosynthetic function.

In our experiments for *Tr. durum* the maximum expression levels were for RbcS and minimum for PEPC. Differences were 23-63 folds, depending on the sample (Figure 5).

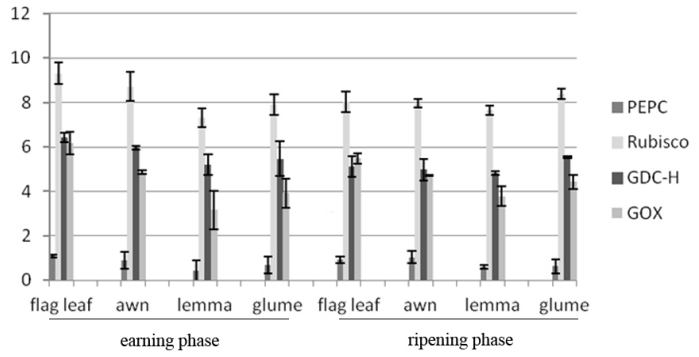


Figure 5: Normalized relative expression level of Rubisco, PEPC, GDC-H and GOX genes in *Tr. durum* samples, in log2 scale. The samples were collected from different photosynthetic active tissues at 2 different developmental phases (earning and ripening). The average of 3 biological repetitions ± 95% confidence interval is presented.

For RbcS in the earning phase the relative expression was higher in the flag leaf comparative to the ear components, but at the ripening phase, for all the samples, the expression was approximately at the same level. The situation is different for PEPC - for majority of samples in both phases the expression level is equal or lower compared with the flag leaf. Greater expression than in the flag leaf was detected only for awn at the ripening phase (1,13 fold). Taking into account the fact that in C_3 plants many PEPC isoforms with other functions (housekeeping functions) are active than those of CO_2 fixation and that the designed primers may be complementary to many of them it is hard to conclude about the expression level of this gene in *Tr. durum* ear components.

For the expression of GDC-H and GOX genes in *Tr. durum* we registered intermediate values compared with RbcS and PEPC. In the leaf the GDC-H expression was greater than in the ear components for both phases, with only exception for the glume at the ripening phase (1,3-fold higher expressions than in the leaf). This means that photorespiratoric cycle is present and active at the considerable level but less active in the ear components than in the flag leaf (Figure 5). Protein H of GDC complex is not involved directly in decarboxylation of glycine but acts as a scaffold for the rest of the three subunits (P, T and L) and it was demonstrated that the increased activity of this protein increases the activity of entire GDC complex [22]. Elevated CO_2 flux through photorespiration cycle leads to the increased photosynthesis rates and 30% higher biomass accumulation. These results were obtained after overexpression of GDC-H from *Flaveria pringley* (C_3) in *Arabidopsis* [26]. The positive effect was explained through photosynthesis blocked by photorespiration metabolites and their catabolism increase reduces this block. Glycolate oxidase (GOX) is one of the key enzymes of photorespiration cycle, it is active in peroxisomes where oxidation of glycolate to glyoxylate occurs. In order to characterize how much active, the photorespiration cycle in ear components and in the flag leaf is - we determined the relative expression of this gene. Obtained data revealed that in all the ear components expression level of GOX was lower than in the flag leaf. During the earning phase in awn, lemma and glume it was 3, 8 and 5-fold lower than in the flag leaf, but at the ripening phase its expression level was 2, 3 and 2-fold lower respectively (Figure 5).

For Triticale samples the expression profile of analyzed genes in general is similar to *Tr. durum*, but there are several peculiarities. The maximum expression level was detected for RbcS but the lowest one for GOX or PEPC, depending on the sample. Registered differences between RbcS and PEPC were greater than those for *Tr. durum*: 600-1700-fold, depending on the sample (Figure 6). Greater PEPC expression than in the flag leaf was registered only for the glume at the earning phase (1,1 fold) and for awn at the ripening phase (1,4 fold). Similar to *Tr. durum* samples the RbcS expression level was greater in the flag leaf compared with the ear components with the exception of awn at the ripening phase (1,1-fold higher expression level than in the flag leaf, Figure 6).

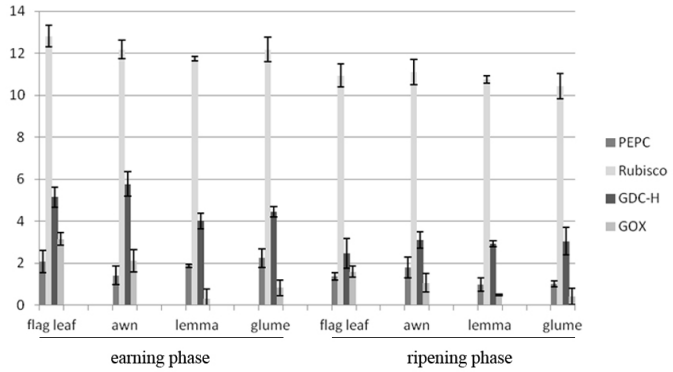


Figure 6: Normalized relative expression level of Rubisco, PEPC, GDC-H and GOX genes in *Triticale* samples, in log scale. The samples were collected from different photosynthetic active tissues at 2 different developmental phases (earning and ripening). Is presented the average of 3 biological repetitions ± 95% confidence interval.

Although differences in RbcS relative expression are not so great between samples, at protein level the differences are clearer: in the flag leaf there is more Rubisco than in ear components (Figure 3 A). For GDC-H gene lower expression levels than in the leaf were obtained for lemma and glume, earning phase (2,2 and 1,6-fold respectively). For awn at the earning phase and all ear components at the ripening phase the expression level of this gene was 1,4-1,6 fold higher than in the flag leaf. Greater expression of GDC-H may be associated with the presence of a glycine shuttle, that favor re-fixation of photorespiratoric CO_2. Recently the existence of a CO_2 concentration mechanism was proved, but previously it was only supposed to be present in the intermediate C_3-C_4 plants and it is considered to act as an intermediate step in the evolution of C_4 photosynthesis [10]. In *Flaveria,* C_3-C_4 intermediate plants, the genes associated with photorespiration were induced at higher levels even than in C_3 species and levels of glycine and serine (transport metabolites in CO_2 pump) were higher than in C_3 and C_4 species of *Flaveria.* Authors concluded that there exist three CO_2 fixation pathways acting in parallel in intermediate *Flaveria* species: NADP-ME, NAD-ME and glycine transport from mesophyll cells (where it cannot be de-carboxylate) into the cells surrounding vascular bundles. Here glycine is de-carboxylated and released CO_2 is re-fixed by Rubisco, all process acts as a CO_2 pump [10].

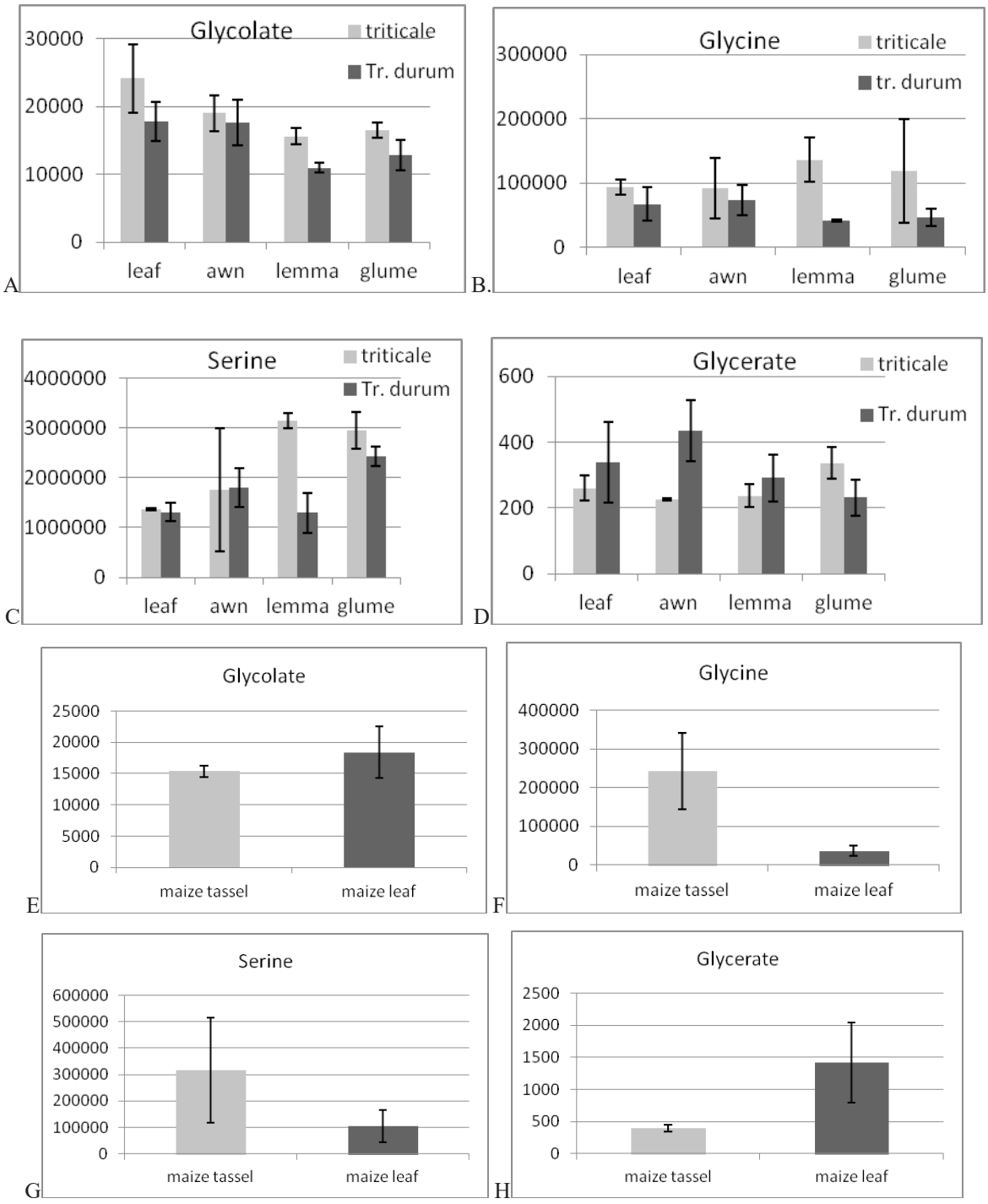
In case of GOX gene in all ear components the expression was lower compared to the flag leaf. Unlike *Tr. durum* in *Triticale* lemma and glume the expression of this gene was even lower than the PEPC expression (Figure 6). From literature data it is known that C_3 - C_4 intermediate species of *Flaveria* do not register intermediate characteristics for photosynthesis and photorespiration genes expression compared to C_3 and C_4 species. Moreover, the abundance of the majority of the transcripts associated with the photosynthesis and the photorespiration were greater than in the C_3 species, the expression of the majority of the genes of Calvin-Benson was C_3 like with exception of RbcL, the expression level of which was significantly lower than in the C_3 species [10].

Metabolites Analysis Through GC-MS

In order to study the metabolite profile in the samples of C_3 and C_4 plants, especially the relative content of photorespiration associated metabolites, we performed the GC-MS analysis. 82 different metabolites were identified in the studied samples, derived from flag leaf and ear components of the greenhouse grown *Triticale, Tr. durum* and maize plants. In our investigations we focused on four main metabolites know to be associated with the photorespiration: glycolate, glycerite, glycine and serine. Glycolate is formed as a result of the photorespiration at the chloroplast level, is transported to the peroxisomes and metabolized to Glycine. Glycine is shuttled back to mitochondria and metabolized to Serine that is shifted back to peroxisome and metabolized to Glycerite. The last one is transported to the chloroplast and fuels the Calvin Cycle.

The values presented in charts in Figures 7-8 are relative, based on the relative and untargeted GS-MS quantification. In the first experiment we analyzed samples derived from greenhouse grown maize, *Triticale* and *Tr. durum* plants at the atmospheric CO_2 concentrations. We used the common stock of grinded samples for metabolites analyses.

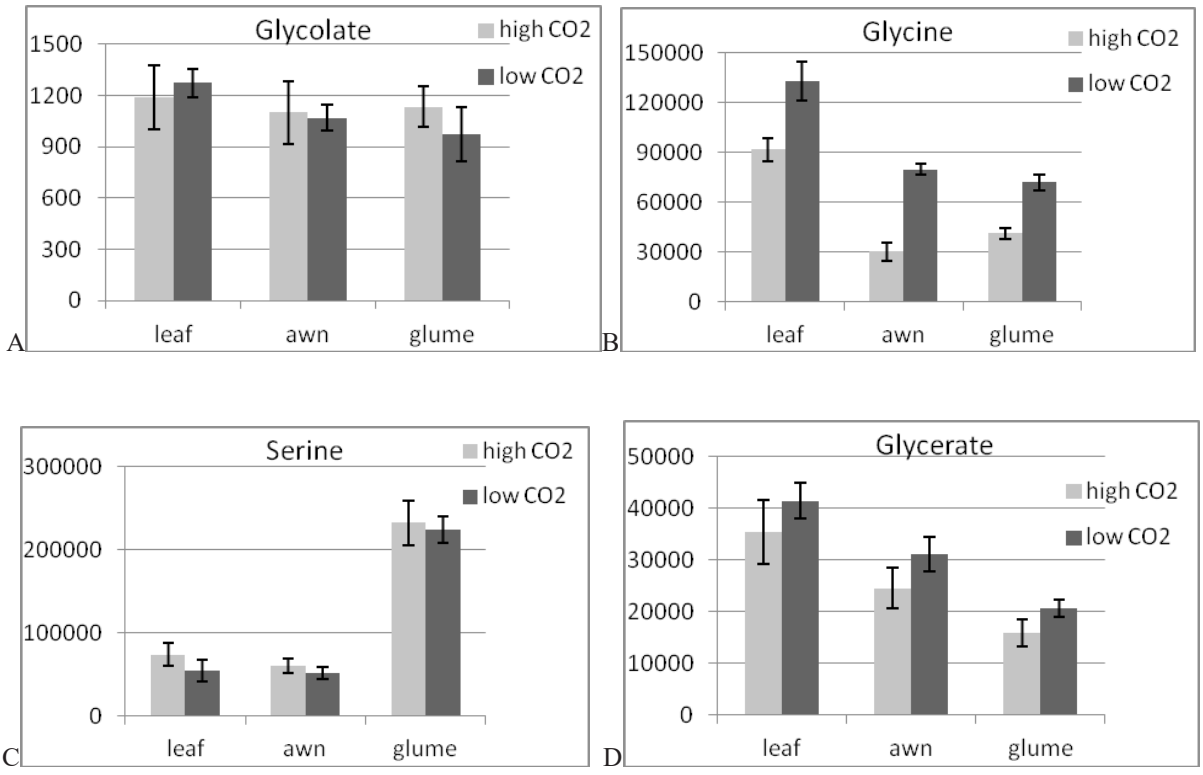
For *Tr. durum* and *Triticale* samples the greatest amount of Glycolate was registered for the flag leaf compared with the ear components (Figure 7 A). Only in case of *Tr. durum* its relative amount was at the same level in the flag leaf and awn. The same trend was registered in case of the maize samples - approximately the same amount of glycolate in the leaf and in the tassel samples (Figure 7 E).



Figures 7(A-H): Relative content of four main metabolites associated with photorespiration (glycolate, glycine, serine and glycerite) in C_3 cereals (*Triticale* and *Tr. durum*) and maize (C_4), determined through untargeted GS-MS analysis. The material was harvested from greenhouse grown plants at ripening phase. Is presented the average of 3 biological repetitions ± 95% confidence interval.

Glycine is the first amino acid that accumulates when the photorespiration cycle is active [23]. For *Triticale* ear samples its content was higher compared with the flag leaf. The same is true for the maize samples - more glycine in the tassel compared with the leaf (Figure 7 F). In case of *Tr. durum* samples, the situation is different: in the ear components the content of Glycine is equal or lower than in the flag leaf (Figure 7 B). Interesting results were obtained for Serine. This amino acid is one the final products of photorespiration cycle obtained after decarboxylation of glycine in mitochondria by GDC complex. The content of serine in the ear components of *Tr. durum* and *Triticale*, with exception of lemma for *Tr. durum*, is greater than in the flag leaf (Figure 7 D). The same situation was registered for the maize samples - more serine in the tassel compared with the leaf (Figure 16 G). Glycerite, the last metabolite of photorespiration cycle, in the cereals samples was at the same level or more abundant in ear components than in the flag leaf (Figure 7 D). In case of maize samples, it was more abundant in the leaf, compared with the tassel (Figure 7 H).

Taking into account the greater relative amount of Glycine, Serine and Glycerite in some of the ear components compared to the flag leaf it is possible to suppose the presence of a Glycine pump, similar to C_3 - C_4 intermediate species. This phenomenon may be explained through the presence of a glycine pump similar to C_3 - C_4 intermediate species of *Flaveria*, where glycine from mesophyll cells is transported to the cells surrounding vascular bundles and is de-carboxylated there [10]. This mechanism of CO_2 concentration is considered as the first step in C_4 evolution [27,28]. In our second experiment we decided to analyze the same four metabolites but in low/high CO_2 atmosphere where photorespiration was induced or suppressed. For that purpose, from greenhouse grown *Triticale* plants, we cut the ears at ripening phase (bellow the flag leaf) and placed in a glass with water in a special adapted chamber, where the CO_2 content is maintained at a certain level.



Figures 8(A-D): Relative content of four main metabolites associated with photorespiration (glycerite, glycolate, glycine and serine) in *Triticale* ears acclimated in low CO_2 (150 ppm, 22°C, 80% humidity) and high CO_2 (2000 ppm, 22°C, 80% humidity) chamber. The plants were grown in greenhouse conditions and harvested ears at ripening stage were placed in a glass with water, in special adapted chamber with low/high CO_2 atmosphere. Metabolites analysis was performed through untargeted GS-MS analysis. The material was harvested from greenhouse grown plants at ripening phase. Is presented the average of 3 biological repetitions ± 95% confidence interval.

Almost no differences were registered for Glycolate relative content, for analyzed flag leaf and ear samples in low and high CO_2 (Figure 8 A). In high CO_2 (suppressed photorespiration) Glycine was more abundant in flag leaf comparing to the ear components. In low CO_2 atmosphere (induced photorespiration) the relative amount of Glycine was greater for each sample, than in high CO_2, but

however, the same trend was maintained- more Glycine in the flag leaf than in the ear components (Figure 8 B). Based on these data it is possible to conclude that photorespiratory cycle is present in the cereals ear components but is less active comparing to the flag leaf.

In case of Serine we registered higher amounts in glume and much lower in the rest of samples for low and high CO_2. For Glycerite we registered the same tendencies as for Glycine: more abundant in low CO_2 conditions for the flag leaf comparative to the ear (Figure 8).

Conclusion

1. The active components of the ear registered increased levels of PEPC activity, with maximum values for glume and lemma. Determination of the relative amount of several key enzymes for photosynthesis and photorespiration (PEPC, RbcL, GDC-H) revealed and abundant level of RbcL in the flag leaf of cereals and of the PEPC in maize leaf. In the ear components were registered traces of PEPC and the amount of RbcL was lower compared to the flag leaf. In the maize leaf was not registered the presence of GDC-H, but these proteins were abundant in the flag leaf of cereals and in lower quantities in the ear components;
2. Carbon isotopes discrimination registered a clear difference between the maize leaf and cereals ear, leaf. The ear components had the same signature as the flag leaf of cereals.
3. GC-MS analysis of the metabolites associated with photorespiration (glycolate, glycerite, glycine and serine) revealed a higher content of serine in the ear components compared to the flag leaf. The same peculiarity was registered for glycine, but only for the ear of *Triticale*. Similar profile of serine and glycine was registered for the maize C_4 leaf and in the tassel the content of these metabolites was higher compared to the leaf;
4. Determination of the relative expression level of RbcS, PEPC, GDC-H and GOX evidenced peculiarities for the photosynthetic active ear components. The most abundant expression was registered for RbcS and minimum for PEPC. The RbcS was more intense expressed in the flag leaf at earning phase of cereals, but in the waxy-milk phase its expression level was similar in the leaf and ear components. For PEPC higher expression was detected in awn, ripening phase. For the rest of ear components its expression level was lower comparing to the flag leaf. The GDC-H gene had more or less similar expression levels in the ear components and flag leaf in *Tr. durum*, but for *Triticale* the highest expression level was detected for the awn. The highest GOX gene expression was detected in the flag leaf and lower levels of expression for the ear components.

General Conclusion

The obtained results demonstrate that the ear of cereals, covering 10-70% from all plant photosynthesis, cannot be considered as an organ with only C_3 type of photosynthesis. There are present also structural and functional elements of C_4 photosynthesis, that may ensure the re-fixation of the CO_2 released from respiration and photorespiration.

As a proof is the lack of apparent photorespiration, but also the presence of an active photorespiration cycle. This complex mechanism that combines elements of C_3 and C_4 photosynthesis, with different expression levels, place the ear in an intermediate zone, similar to C_3 - C_4 plants. The lack of apparent photorespiration and the concomitant presence of the photorespiration cycle in the ear may serve as pomp that ensure with CO_2 the pentose phosphate cycle and increase the efficiency of photosynthesis.

It is possible to suppose that during the evolution of C_4 plants this pomp was substituted by the C_4 cycle, where the CO_2 fixation is performed in the mesophyll cells by PEPC, an enzyme with only carboxylation capacity, different from Rubisco. These peculiarities may open new opportunities for biotechnological use of evidences mechanisms in order to increase the photosynthesis efficiency and productivity of cereals.

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