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Long-lasting low temperature pretreatment of soybean seeds enhance plant field performance and content of free metabolites

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Abstract

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The current study presents results from three years experimental work focused on evaluation of climate plasticity and content of free amino acids, mono- and disaccharides and fatty acids of three soybean cultivars by using an innovative approach of long-lasting low temperature pretreatment of soybean grains before sowing. The results from field performance of plants grown from pretreated and non-treated seeds were taken based on five morphological indicators. Expression and metabolomic profiles of leaves, green seeds and mature seeds of plants grown from low temperature pretreated and not-treated seeds were assessed by real time quantitative reverse transcription *polymerase chain reaction* (qRT-PCR) and gas chromatography/mass spectrometry (GC/MS). The all three years collected data from the morphological analyses, expression profiles and content of metabolites confirmed that the plants grown from low temperature pretreated seeds possess better field performance, enhance content of free amino acids, sugars and fatty acids in green and mature seeds. The obtained expression profiles of selected genes correlated with the content of amino acids and sugars. The obtained results in the present study could benefit legume crops researchers, plant breeders, farmers and processors of soybean.

Keywords: soybean; long-lasting pretreatment of seeds; morphological analyses; expression analyses; metabolomic analyses

Introduction

Soybean (*Glycine max*) is one of most famous worldwide used crop, which ensures large amounts of vegetable protein and edible oil for humans and animals. The vegetable protein in this crop is 3 to 4 times more and reach of essential amino acids than some other basic cereal crops such as wheat, corn and barley, and 1.5 to 2 times more protein than other legumes (Ge et al., 2016). The modern soybean breeding is focused on new varieties, which possess high and stable yields, and grains with economic qualities, as the high content of crude protein and oil. The very important issue of soy protein is its excellent quality, characterized with the presence of all the essential amino acids necessary for the building of protein in animals and humans. Soybean cropping is influenced by environmental factors especially drought. This is the main reason the efforts of the breeders to be directed for the creation of local cultivars (Iantcheva et al., 2021). Bulgaria is situated at the East-South Europe and its climate is moderately continental and different from other parts of continent.

The countries in the Danube region, are suitable for soybean production and among them Bulgaria possesses the optimal ecological and production conditions for its cultivation. The main factor necessary for crop production as a soil, heat and light regime are suitable for soybean and the factor which limits its production which could reduce the yield to 40% are the continues droughts in the periods of pod formation and grain filling (Naydenova et al., 2022). In Bulgaria efforts of the breeders are related to programs for development of local cultivars adapted to the specific agro-climatic environment. The exiting Bulgarian soybean cultivars in combination with optimal agro technology are a prerequisite for good production results even in the conditions of no irrigated conditions. The modern soybean research aims to study plant genotype and its interaction with changes in the environment. Such complicated research is focused on large-scale experiments based on structural and functional genetics. In innovative research such complex studies are named "omics and include genomics, transcriptomics, proteomics and metabolomics. These "omics" approaches are applied in many research groups working with model and crop plants, including soybean. In the last two decades many data were accumulated from the performed "omics" experiments and as a result extensive databases for soybean have been created (Soybean Knowledge Base, http://soykb.org); Soybean transcription factors database http://casp.rnet.missouri.edu/soydb/; soybean metabolome database http://soymetdb.org (Deshmukh et al., 2014). Soybean research investigations are aimed at increasing the yield and quality of vegetable protein. The published results up to date concern soybean varieties developed in different countries related to the specific climatic requirements and needs of agriculture (Mandich et al., 2014; Zajac et al., 2017).

In the three-year period 2019-2021 we conducted an innovative approach of pretreatment of soybean seeds with low temperature before sowing. The experiment was performed with two Bulgarian soybean *cvs*. (Avigea and Richy) compared to French *cv.* (Izidor). In our experimental work, the cold pretreatment of soybean seeds with low temperature (2-5°C), was for two periods 12 days – called 'treated control' and 22 days – called 'treated'. Our investigation was focused on how low temperature pretreatment of soybean seeds later on affected plant field performance and plants morphology, transcript level of selected genes, quantity of free amino acids, fatty acids and sugars detected in green and mature seeds collected from plants grown from pretreated seeds. All these parameters were compared to soybean plants grown from 'non-treated' seeds. The experimental results from first two years are described in (Iantcheva et al., 2021). In the current study we present the results from third year, summarize and discuss the data of three years experiment of low temperature pretreatment of soybean seeds before sowing. The results are focused on field performance of plants, transcript profiles of selected genes, and quantity of free metabolites mentioned above. All these data are compared to plants grown from non-treated seeds.

Material and Methods

Plant material and field growth conditions

The three years study was conducted at the field of Experimental Station for Soybeans and grain crops, Pavlikeni situated in North Central Bulgaria, (43°23′N, 25°32′E, 144 m above sea level). The soil type is determined as moderately leached chernozem, with a humus horizon thickness of 40-50 cm. The soil (pH) is neutral with humus content of 3-4%; soil porosity is 47% and quench humidity is 15.2% (Naydenova & Vasileva, 2019). The experimental work was performed with two Bulgarian soybean cultivars with commercial value, belonging to maturity group I – Avigea, Richy and French cultivar Izidor (used as a standard).The field experiments were conducted, through the method of long plots without irrigation and chemical fertilization. The plots were organized in rows, with row spacing of 70 cm and sowing rate of 40 000 seeds/decare (Georgiev & Todorova, 2018). The seeds were sown between 25-30 April of each year.

Plant morphology was described by biometric characteristics on the selected indicators: plant height (cm), number of pods, number of seeds per plant, seed yield from plant (SY, g), absolute mass of seeds (m 100 g). Each year of the experiment the value of indicators was taken, during the phenophase R8.

Low temperature pretreatment of seeds

The laboratory and field experiments were performed with pretreated and non-treated seeds. Pretreated seeds: seeds of the *cvs.* Avigea, Ritchy and Izidor were pretreated with low temperature in controlled conditions before sowing. Treatment scheme: All seeds were placed in petri dishes with wet filter paper for 2 days at 2°C; 10 days at 4°C; After this period these are called 'treated control' were transferred in a controlled chamber with day temperatures of 16°C and night temperatures of 8°C with a photoperiod of 16 hours day and 8 hours night for a period of 10 days. The seeds called 'treated' were incubated for another 10 days at 5°C. After pretreatment the seeds and seedlings were transferred to the experimental field in Pavlikeni and they were planted together with the 'non-treated' on the field as described above.

Gene expression analysis

In order to investigate transcript profiles we selected genes highly expressed in the stage of development of soybean R5-R7 (www.soybean.org). Two of selected genes were related to abiotic stress: *Glyma05G185500*, which encodes alkaline/neutral *Invertase* involved in sucrose catabolic process; *Glyma13G162800,* which encodes a H(+)-translocating (pyrophosphate-energized) inorganic pyrophosphatase (H(+)-PPase) located in the vacuolar membrane. The rest of selected genes for expression analyses were related to amino acids transporters. Chenh et al. (2016) divided the amino acid transporters into several groups. For the present study we selected transporters from 3 groups: ANT group (aromatic and neutral AA transportes): *Glyma19G39060*, which encodes an amino acid transporter that transports aromatic and neutral amino acids, IAA, and 2,4-D; LHT group (Lysine/Histidine transporters): *Glyma13G245000*, which encodes transmembrane amino acid transporter, orthologue of AT1G47670, Lys/His transporter 7; ProT group (proline transporters): *Glyma18G031300*-orthologue AT2G39890, which encodes proline transporter 1.

Plant material used in expression analyses consisted of leaves and green seeds (GS) collected at stage R5-R7 and mature seeds (MS). Total RNA was extracted from the respective tissues with the RNA Plant Kit (Eurex). 1µg of total RNA was reverse transcribed with the First Strand cDNA Synthesis Kit (Bio-Rad). Relative expression levels were determined with the 7300 Real-Time qPCR System (Applied Biosystems, http://www.appliedbiosystems.com). The qRT-PCR was carried out in a total volume of 20 µL containing 5 µL of cDNA, 0.5 µL gene specific primers (10 µmol/L), 10 µL SYBR Green Mix (Eurex) and 4 µL of RNase free ddH_2O. The PCR conditions were as follows: 95°C for 5 min, followed by 60 cycles of 95°C for 15 s and 60°C for 30 s. Two different reference genes (*ACTIN* and *UBIQUITIN10*) were used for normalization. For each set of samples three biological repeats, each with three technical repeats were used for transcript data. The transcript profile displayed in figures is a pool of three biological repeats.

Metabolomic analyses

The two fractions obtained – polar (amino and organic acids, carbohydrates) and non-polar (saturated and unsaturated fatty acids, sterols) were determined by Gas Chromatography-Mass Spectrometry (GC-MS) in green and mature soybean seeds.

Briefly, 50.0 mg lyophilized material from each sample were subjected to the following procedure: 500.0 µL methanol, 50.0 µL ribitol and 50.0 µL n-nonadecanoic acid (internal standards in concentration 1 mg/mL to quantify of polar and non-polar metabolites, respectively) were added, then the resulting mixture were heated using a thermo shaker TS-100 (Analytik Jena AG, Germany) 30 min/70°C/300 rpm. 100.0 µL water and 300.0 µL chloroform were added after cooling down to room temperature, then the mixtures were centrifuged (5 min/22°C/13000 rpm, Beckman Coulter). The upper phase lower phase was designed for the analysis of polar compounds, whereas the lower phase was for study of the non-polar metabolites. The two phases obtained were vacuum-dried at 40ºC in a centrifugal vacuum concentrator (Labconco Centrivap).

In order to extract the saturated and non-saturated fatty acids, 1.0 mL 2% H_2SO_4 in MeOH was added to the dried residue of fraction "non-polar metabolites", then the mixture was heated on a thermo shaker (1 h/96ºC/300 rpm). After cooling to the room temperature, the resulting solution was extracted with n-Hexane (3x10.0 mL). Organic layers combined were vacuum-dried at 40ºC in a centrifugal vacuum concentrator (Labconco Centrivap).

Prior to analysis by GC-MS, samples were derivatized by the following two procedures:

First, a 300.0 µL solution of methoxyamine hydrochloride (20.0 mg/mL in pyridine) was added to a fraction of "polar metabolites", and the mixture was heated on a thermo shaker 1h/70ºC/300rpm. After cooling, 100.0 µL N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA) were added to the mixture, then heated on a thermo shaker 40min/70ºC/300rpm. Finally, 1.0 µL from the solution was injected in the GC-MS.

Second, 100.0 µL pyridine and 100.0 µL BSTFA were added to a fraction of "non-polar metabolites", and then heated on a thermo shaker 45 min/70ºC/300 rpm. Then, 1.0 µL from the solution was injected in the GC-MS.

GC-MS analysis was carried out using a gas chromatograph 7890A (Agilent) coupled to a mass selective detector 5975C (Agilent) and HP-5ms silica-fused capillary column coated with 0.25 µm film of poly (dimethylsiloxane) as the stationary phase (Agilent), 30 m×0.25 mm (i.d.). The oven temperature program used was as follows: initial temperature 100°C for 2 min, then 15°C/min to 180°C for 2 min, then 5°C/ min to 300°C for 10 min, run time 42 min. The flow rate of the carrier gas (Helium) was maintained at 1.2 mL/min. The injector and the transfer line temperature were kept at 250°C. The temperature of the MS source was 230°C. The injections were carried out in a splitless mode; the injection volume was 1 µL.

In order to calculate the relative retention indices (as Kovàts index, RI) of each compound, a mixture of aliphatic hydrocarbons from C_{10} to C_{40} (Sigma) was injected under the above temperature program. The identification of the metabolites was obtained by comparing the retention times and RI with those of authentic compounds and the spectral data obtained from The Golm Metabolome Database-GMD (Hummel et al., 2010) and National Institute of Standards and Technology (NIST 08) libraries (Manion et al., 2015).

Statistical analyses

Data represent the mean ± SE or SD and in average were analyzed with $n \geq 3$. The One-way analysis of variance (ANOVA) (Holm-Sidak) and Student (*t*-test) statistical tests were applied to estimate the difference between all the variants (differences were considered statistically significant at the $p < 0.05$ level). The significance of the genotypic variance for the experimental period was established by ANOVA two-factor analysis of variance.

Results and Discussion

The three experimental years were characterized with different rainfall regimes in the vegetative and reproductive phases of crop development. The first experimental year (2019) was characterized with very favorable conditions in the periods of flowering and pod formation. During the flowering phase the monthly precipitation in June 2019 was 149.0 mm, and the distribution of precipitation over ten days was also even. In the second year 2020, there was drought, the stage of pods development took place in the conditions of severe drought with precipitation amounts of 6.4 mm in July and 36.4 mm in August. The most unfavorable agrometeorological conditions were detected at 2021. During the period April-September, 281.8 mm rainfalls fell. In April they were 8 mm above the norm, in May they were above the norm by 34 mm, and in the remaining 4 months they were much below the norm, especially in July – 10.1 mm. The reproductive period R1-R8 (flowering – ripening), is critical in terms of water availability and took place with very low moisture supply, which caused extremely unfavorable effect on the yield. According to the water availability for the period IV-VIII, 2021 is characterized as extremely dry, with precipitation much below the norm and with unfavorable distribution by months. In the three years experimental period, 2021 was the second dry year together with 2020. The monthly precipitations for the three consecutive years are presented in Table 1.

Table 1. Rainfall sums for three experimental years 2019-2021. by months, during the vegetation period of soybean

Months Years	IV	V	VI	VII	VIII	IX
2019	94.4	41.8	149.0	77.8	8.6	2.4
2020	20.2	47.3	99.1	6.4	36.4	22.4
2021	51.8	104.2	65.7	10.1	26.4	23.6

Evaluation of field performance according to 5 indicators of plants grown from seeds with or without low temperature pretreatment

The collected results from the evaluation of morphological indicators during the first two experimental years based on selected indicators illustrates that the plants grown from the treated seeds ‘treated’ and ‘treated control’ possessed higher values of the studied indicators. This difference was significant ($p < 0.05$; $p < 0.01$) in terms of the number of pods, the number of seeds per plant and the mass of seeds per plant (Iantcheva et al., 2021).

The third experimental year was worst in respect to summer drought and the values of the selected indicators were lowest than those in the 2019 and 2020 experimental years. The comparison of the results based on the selected indicators illustrates that the plants grown from the pretreated seeds, ‘treated control’ and ‘treated’, showed higher values

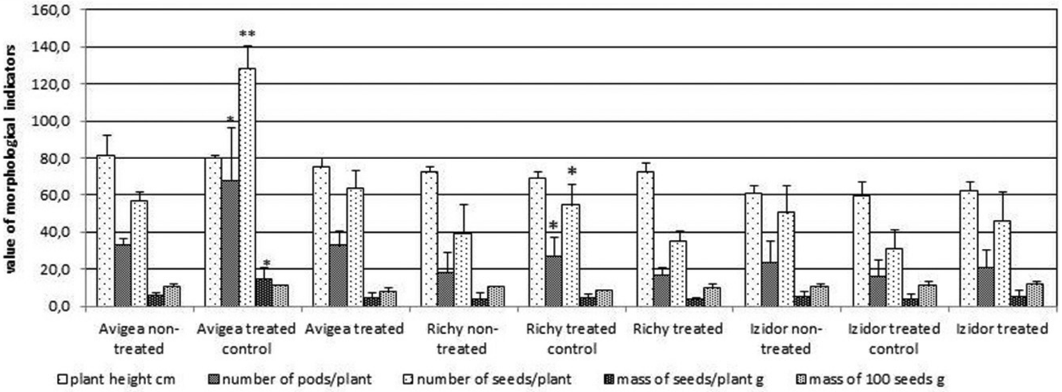

Fig. 1. Comparative analysis of 6 indicators of plants grown from seeds pretreated with low temperature – ‘treated’, ‘treated control’ and ‘non-treated’ – in the third experimental year, 2021. Data represent the mean ± SD. The One-way ANOVA (Holm-Sidak) statistical test was applied to assess the difference between all the variants. Asterisks denote statistically significant differences of treated and treated control compared to ordinary * $p < 0.05$; $p < 0.01$**

Table 2. Results from Two-factors analysis of variance of the morphological indicators in three years field experiment for *cvs*. Richy and Izidor. NS – not significant

Plant height cm	Richy non-treated	Richy treated control	Richy treated	Significance of ecological factor	Significance between treatment	Izidor non-treated	Izidor treated control	Izidor treated	Significance of ecological factor	Significance between treatment
2019	105,6	108,4	100,6	P < 0.001	NS	78,4	61,8	59,25	NS	P < 0.05
2020	72,1	80	75,6			75,2	67,6	53,6		
2021	72,6	69,4	72,8			61,2	59,6	62,4		
Number of pods/ plant	Richy non-treated	Richy treated control	Richy treated	Significance of ecological factor	Significance between treatment	Izidor non-treated	Izidor treated control	Izidor treated	Significance of ecological factor	Significance between treatment
2019	53	143,2	134,8	**P < 0.01**	**P < 0.05**	51	178,8	187,5	**P < 0.01**	**NS**
2020	93,5	142,66	242,66			65,1	149,3	97,33		
2021	18,2	27,4	16,8			23,6	16,2	20,8		
Number seeds/ plant	Richy non-treated	Richy treated control	Richy treated	Significance of ecological factor	Significance between treatment	Izidor non-treated	Izidor treated control	Izidor treated	Significance of ecological factor	Significance between treatment
2019	110	279	288,8	**P < 0.01**	**P < 0.05**	97,6	406,4	397,75	**P < 0.01**	NS
2020	171,9	255,66	263,33			121,9	287	205		
2021	39,2	54,8	35,2			50,6	31,2	46,2		
SY, g	Richy non-treated	Richy treated control	Richy treated	Significance of ecological factor	Significance between treatment	Izidor non-treated	Izidor treated control	Izidor treated	Significance of ecological factor	Significance between treatment
2019	13,56	31,58	28,18	**P < 0.001**	**P < 0.05**	14,92	86,18	72,35	**P < 0.01**	NS
2020	26,6	33,63	38,9			21	49	32,43		
2021	4,334	4,938	3,754			5,538	3,934	5,33		
m 100, g	Richy non-treated	Richy treated control	Richy treated	Significance of ecological factor	Significance between treatment	Izidor non-treated	Izidor treated control	Izidor treated	Significance of ecological factor	Significance between treatment
2019	12,5	11,1	9,9	**P < 0.05**	**NS**	15,1	21,3	18,1	**P < 0.001**	**P < 0.05**
2020	12,5	12,7	14,7			15,1	17,6	15,3		
2021	10,9	8,9	10,4			10,7	11,6	12,3		

of the studied indicators in comparison to 'non-treated' for the Bulgarian cvs. Avigea and Richy, but the cv. Izidor the values of the indicators number of pods, number of seeds/ plant, mass of seeds/plant was higher or equal in 'non-treated' seeds compared to treated with the exception of the indicator mass of 100 seeds (Figure 1). According to the results of the two factorial analysis of variance, we detected differences for the treatment and for the experimental period and they were significant at ($p < 0.05$, $p < 0.01$, $p < 0.001$) for the following traits plant height, number of pods/plant, number of seeds /plant, seed yield per plant, mass of100 seeds for both cvs. Richy and Izidor (Table 2). For cv. Richy the ecological factor was more important, there was significant difference for all 5 indicators, while the factor treatment was significant only for 3 indicators. In the case of cv. Izidor the treatment was less significant, only for two indicators there were significant differences, but for ecological factor significance was established for 4 indicators (Table 2).

The conducted approach of pretreatment of seeds with low temperature was traced during three consecutive years and was performed in order to test the ability of seeds to germinate in the conditions of abiotic stress /low temperature/ and grow strong plants able to produce seeds of the desired quantity and quality. In Bulgaria the optimal condition for sowing the soybean is when soil temperature is about 10°C, which corresponds to the last 5-10 days of April. In the beginning our intent was to use pretreatment of seeds with low temperature in order to simulate sowing conditions at the beginning of April. In Bulgaria, early planting of soybean would allow early or mid-early varieties to pass the critical phase of flowering and filling of pods not in the condition of strong summer droughts, which limit the yield of crop. As a benefit from low temperature pretreatment we observed better filed performance of plants grown from pretreated seeds as well as higher content of free metabolites which affected quality of seeds. Recent investigation on long-term cold treatment of lupine seedlings (7°C) was published. The authors found that seeds well germinated at 7°C and demonstrated higher activity of α-amylase and higher levels of gibberellins, IAA and kinetin. Germination ability at low temperature correlated with dehydrogenase activity and membrane permeability (Plazek et al., 2018).

During three years of field experiment, the pretreated seeds showed lower germination ability than non-treated. Vieira and co-authors in 2010 published results of short cold pretreatment of soybean seeds from one cultivar with a temperature of 10°C for short period of treatment of 5 days. The authors confirmed seed germination ability of 84-94%. Vinkovic et al. (2007) investigated the effect of fungicides, biostimulants and proline pretreatment on seeds vigor and germination in the condition of low temperature in maize and soybean. Our three years, data of long-lasting low temperature pretreatment confirm the decrease of seed germination ability of ‘treated controls’ at the range of 10 to 15% for cv. Avigea and 5% for cv. Richy. In case of cv. Izidor the germination ability of ‘treated controls’ was higher with 4

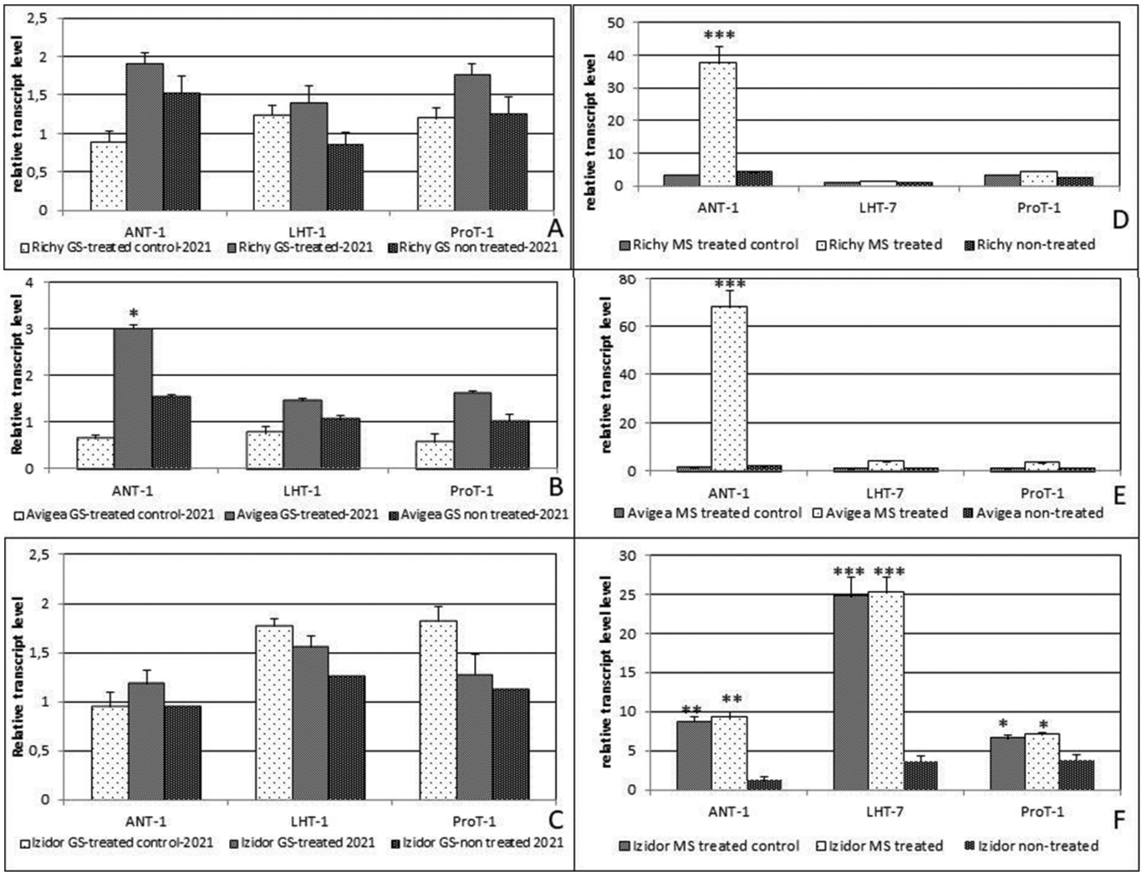

Fig. 2. Relative transcript level of the set of sleeted genes /*ANT-1; LHT-7; Prot-1*/ in green seeds (GS) and mature seeds (MS) of plants grown from ‘treated’, ‘treated control’ and ‘non-treated’ seeds of the three investigated cultivars in 2021.: A-transcript level in GS of *cv*. Rhichy; B-transcript level in GS of *cv*. Avigea; C- transcript level in GS of *cv*.Izidor; D – transcript level in MS of *cv*. Richy; E-transcript level in MS of *cv*. Avigea. F- transcript level in MS of *cv*. Izidor. Data represent the mean ± SE. The One-way ANOVA (Holm-Sidak) statistical test was applied to estimate the difference between all the variants. Asterisk denote statistically significant differences

$* p < 0.05$;$** p < 0.01$; $*** p < 0.001$

% than in 'non-treated'. The 'treated' seeds possess better germination capacity than 'non-treated' at a range of 3-10% in cvs. Richy and Izidor. Only in the case of cv. Avigea the germination capacity of 'treated' drop down with 10% compared to 'non-treated'. Even with reduced germination ability the plant obtained from pretreated seeds possess high vigor and value of the morphological indicators, number of pods, number of seeds, mass of seeds per plant and mass of 100 seeds (Figure 1).

Evaluation of gene expression data in, leaves, green seeds and mature seeds of plants grown from seeds with or without low temperature pretreatment

The selected set of genes for the expression analyses in 2021 included: one gene which expression indicated response to drought /*H-PPase*/, one gene responsible for catabolism of sucrose *Invertase* and their expression were detected in leaves and GS seeds as a target tissue; three AAs transporters, /*ANT-1*-aromatic and neutral transporter1, *LHT-7* transporter-lysine histidine transporter 7 and *ProT*-proline transporter 1 and GS and MS as a target tissue. The transcript level detected in 2021 in green seeds (GS) of *cv.* Righy was highest in plants grown from 'treated' seeds for three AA transportes /*ANT-1, LHT-7* and *ProT-1*/ followed by 'non-treated' for *ANT-1* and 'treated control' for *LHT-7* and *ProT-1* transporters (Figure 2A). For the second Bulgarian cv. Avigea we found the highest level of transcript profiles for the three AA transporters in GS from plants grown from 'treated' seeds followed by 'non-treated' and lowest in 'treated control' (Figure 2B). The obtained expression profiles in cv. Izidor in GS were different. The highest expression level for *ANT-1* transporter was detected for GS collected from plants grown from 'treated' seeds and equal in 'treated control' and 'non-treated'. The results for the other two AA transporters *LHT-7* and *ProT-1* indicated that the level of expression was highest in GS taken from plants obtained from 'treated control' seeds, followed by 'treated' and lowest in 'non-treated' (Figure 2C). The observed expression profiles in mature seeds (MS) of three cvs. were different than those in GS. In cv. Richy and *cv*. Avigea the expression of *ANT-1* transporter was highest in MS from plants grown from 'treated' seeds and low and almost equal in 'treated control' and 'non-treated' (Figure 2D, E). The expression profiles for the other two AA transporters were almost equal for three types of MS collected from plants obtained from 'treated', 'treated control' and 'non-treated' seeds. For cv. Izidor the expression level for three AA transporters was high or almost equal in MS collected from plants grown from 'treated' and 'treated control' seeds and much low in the 'non-treated' (Figure 2F). In our previous study (Iantcheva et al., 2021) we discuss the

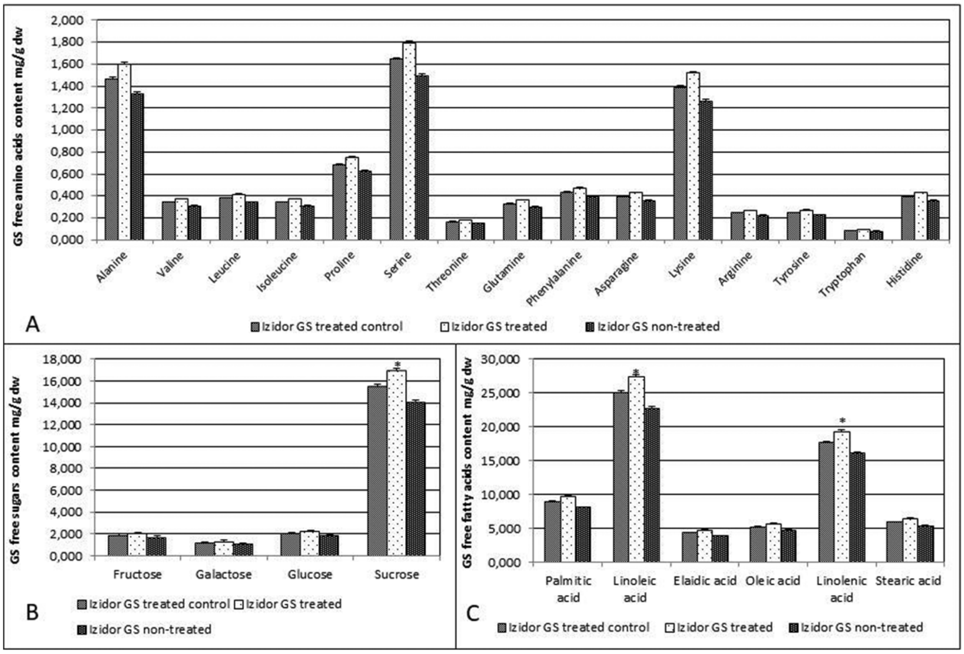

Fig. 3. Content of detected free metabolites (mg/g dw) in green seeds (GS) of cv. Izidor in 2021. A – free AAs; B – free sugars; C – free FAs. Data represent the mean ± SD. Asterisks denote statistically significant differences * $p < 0.05$

correlation of transcript level of *Invertase* and the three AAs transporters in GS and MS and detected content of sugar and free amino acids during the first two years of experiment. In the results of third year we could confirm again this correlation of the transcript level and content of free metabolites.

In the worst environmental conditions during the third year 2021 the transcript level of *H-PPase* detected in leaves were significantly elevated in plants grown from pre-treated seeds 'treated control' and 'treated' than in 'non-treated (data not shown). In the literature from the last 10 years the role of *H-PPase* in plant genome is related to the responses to water deficiency, salt stress, and maintenance of transmembrane

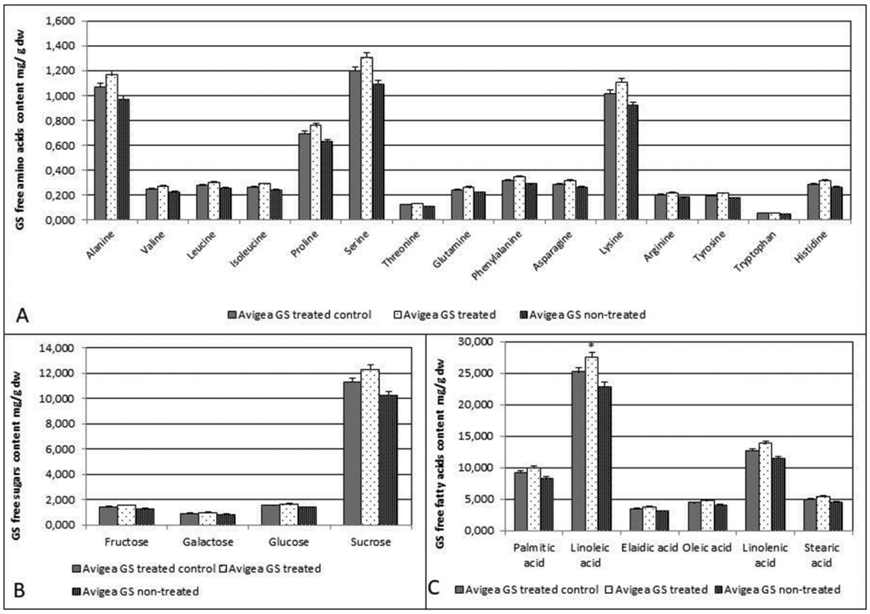

Fig. 4. Content of detected free metabolites (mg/g dw) in green seeds (GS) of cv. Avigea in 2021. A – free AAs; B – free sugars; C – free FAs. Data represent the mean ± SD. Asterisks denote statistically significant differences * $p < 0.05$

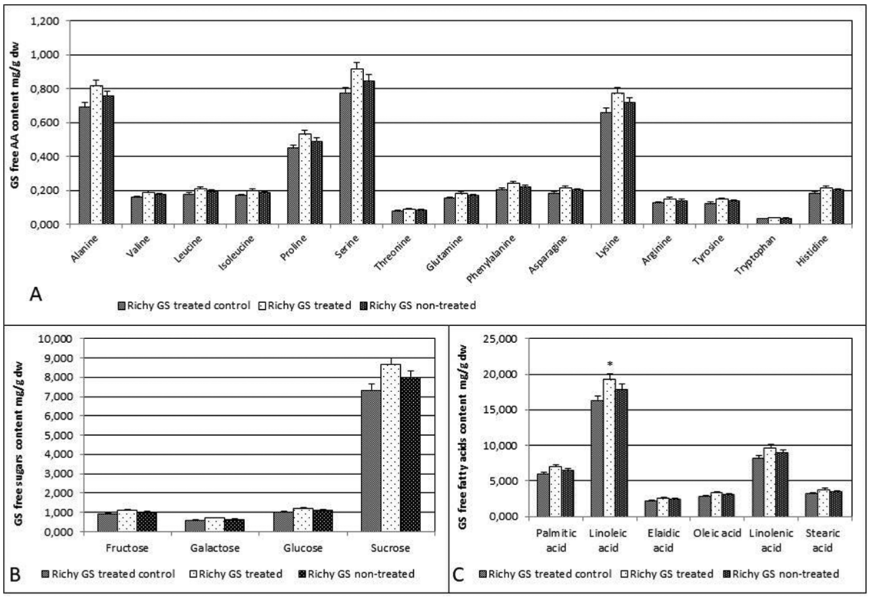

Fig. 5. Content of detected free metabolites (mg/g dw) in green seeds (GS) of cv. Richy in 2021. A – free AAs; B – free sugars; C – free FAs. Data represent the mean ± SD. Asterisks denote statistically significant differences * $p < 0.05$

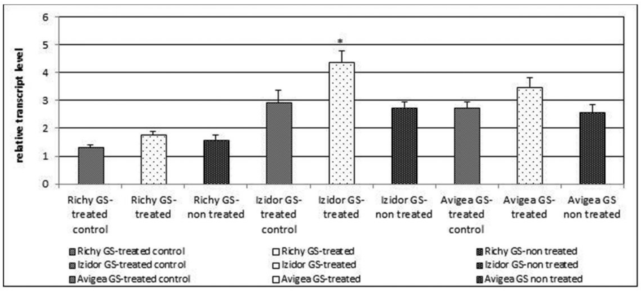

Fig. 6. Relative transcript level of Invertase gene in green seeds (GS) of plants grown from 'treated', 'treated control' and 'non-treated' seeds of the three investigated cultivars in 2021. Data represent the mean ± SE. The One-way ANOVA (Holm-Sidak) statistical test was applied to estimate the difference between all the variants. Asterisk denote statistically significant differences * $p < 0.05$

electrochemical gradient (Schilling et al., 2017; Bhaskaran & Savithramma, 2011). In the condition of severe summer drought in the year 2021, the significantly elevated level of expression of *H-PPase* in 'treated' and 'treated control' leaves indicated better field performance of plants grown from pre-treated seeds. In this respect the investigated approach of long-lasting low temperature pretreatment of seeds before sowing could be a way to enhance the tolerance of plants in condition of drought.

Evaluation of the level of free amino acids, sugars and fatty acids in green and mature seeds of plants grown from seeds with or without low temperature pretreatment

In 2021 in the obtained metabolite profiles of cv. Izidor in GS of plants grown from 'treated' seeds we observed the highest content of all detected free AAs, fatty acids and sugars. These contents were followed by 'treated controls' and the less amount of free amino acids, sugars and fatty acids were detected in GS collected from plants grown from 'non-treated' seeds (Figure 3A-C). Similar results were established for cv. Avigea. The quantities of free AAs, sugars and fatty acids were highest in GS collected from plants grown from 'treated' seeds, followed by 'treated control' and less in 'non-treated' (Figure 4 A-C). In cv. Richy again the detected content of free AAs, sugars and fatty acids were highest in samples of GS collected from plants grown from 'treated' seeds but they were followed from 'non-treated' and less amount of metabolites was detected in GS from 'treated control' (Figure 5 A-C).

In the obtained metabolite profile of 'treated' GS of *cv.* Izidor we observed the highest content of free sucrose (16.935 mg/g dw) compared to 'treated control' (15.524 mg/g dw) and 'non-treated' (14.115 mg/g dw) $p < 0.05$. In the expression analyses of GS in 2021 the level of transcript of *Invertase* gene was high in 'treated' GS of cv. Izidor, (Figure 6; 3B). For cv. Avigea high expression level of *Invertase* was observed in 'treated' GS, followed by 'treated control' and 'non-treated' and this results corresponds to the content of sugar 12. 326 mg/g dw of 'treated'; 11. 299 mg/g dw of 'treated control'; 10. 271 mg/g dw 'non-treated' (Figure 6, 4B). In cv. Richy we observed the same correlation between gene expression and metabolite content of sugar of 'treated' GS, which possessed high value (8.663 mg/g dw) compared to 'non-treated' (8.022 mg/g dw) and 'treated control' (7.349 mg/g dw) (Figure 6, 5B).

The function of gene *Invertase* in plants is responsible for irreversible catalysis of the hydrolysis of sucrose into hexoses (glucose and fructose). Sucrose is the major end-product of photosynthesis and the simple sugars glucose and fructose are used from plants as nutrients, energy sources, and as signaling molecules in the processes of plant development, yield formation and stress responses (Wan et al., 2018; Vargas et al., 2008). The high contents of free sugar and high expression levels of *Invertase* in GS of plants grown from 'treated' seeds in three cvs. tested suppose the enhanced vigor and filed performance in the condition of drought of those plants.

In 2021, the detected content of free fatty acids (FAs), (2 saturated and 4 unsaturated) in GS the three tested cultivars is presented on Figures 3C, 4C, 5C. The content of all 6 fatty acids was high in GS collected from plants grown from 'treated' seeds, followed by 'treated control' in cvs. Izidor and Avigea and for the cv. Richy was higher in 'treated' followed by 'non-treated' and less in 'treated control'.

The content of all detected free AAs, sugars and fatty acids in MS collected from plants grown from 'treated' seeds of the three investigated cultivars was highest, than in MS 'treated control' but no significant difference was found. The content of all detected free metabolites in MS 'non-treated', was significantly less than in 'treated' ($p < 0.05$; $p < 0.01$) (cv. Izidor (Figure 7 A-C); cv. Avigea (Figure 8 A-C); cv. Richy (Figure 9 A-C).

Although the unfavorable environmental condition of 2021, the contents of free AAs, (leucine, histidine, glutamine, lysine, methionine, alanine, proline, valine), sucrose in MS of plants grown from 'treated' seeds in cvs. Izidor were higher than previous two years (2019, 2020) and in cv. Avigea than in year 2019 (Iantcheva et al., 2021). The detected content of unsaturated FAs (linoleic and linolenic) in MS of plants grown from 'treated' seeds in cv. Izidor was significantly higher ($p < 0.05$) than the content detected in year 2019 and 2020. The amino acids are the main form of nitro-

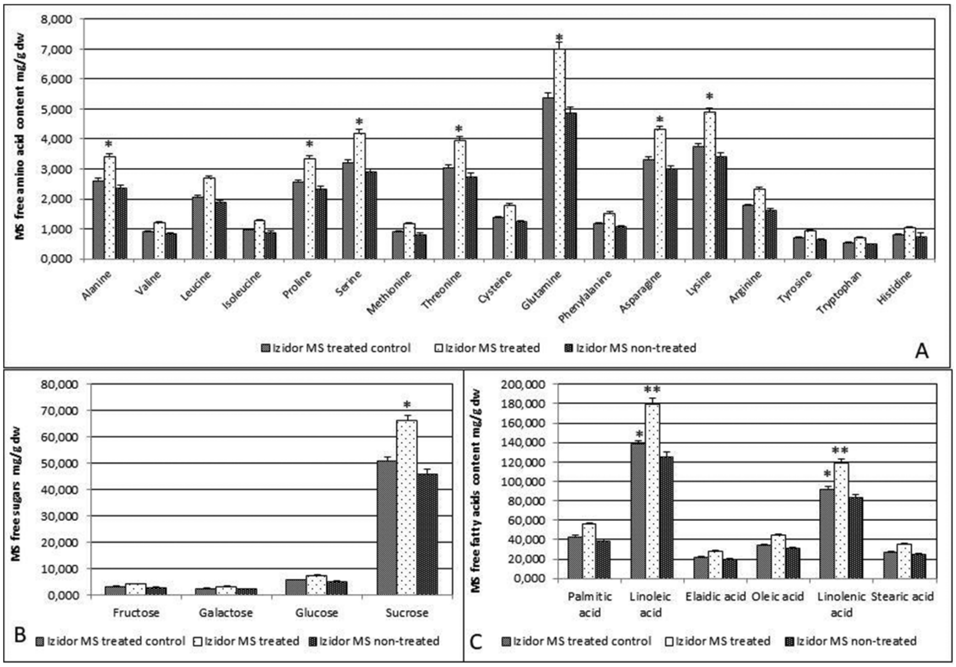

Fig. 7. Content of detected free metabolites (mg/g dw) in mature seeds (MS) in cv. Izidor in 2021. A – content of free AAs; B – content of free sugars; C – content of free FAs. Data represent the mean ± SD. Asterisks denote statistically significant differences *p < 0.05; p < 0.01**

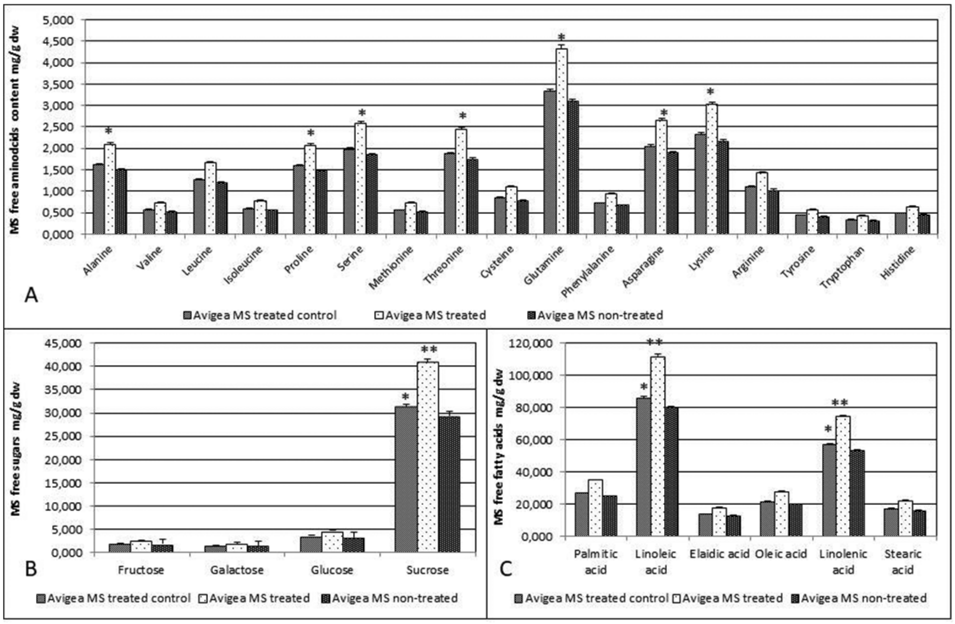

Fig. 8. Content of detected free metabolites (mg/g dw) in mature seeds (MS) in cv. Avigea in 2021. A – content of free AAs; B – content of free sugars; C – content of free FAs. Data represent the mean ± SD. Asterisks denote statistically significant differences *p < 0.05; p < 0.01**

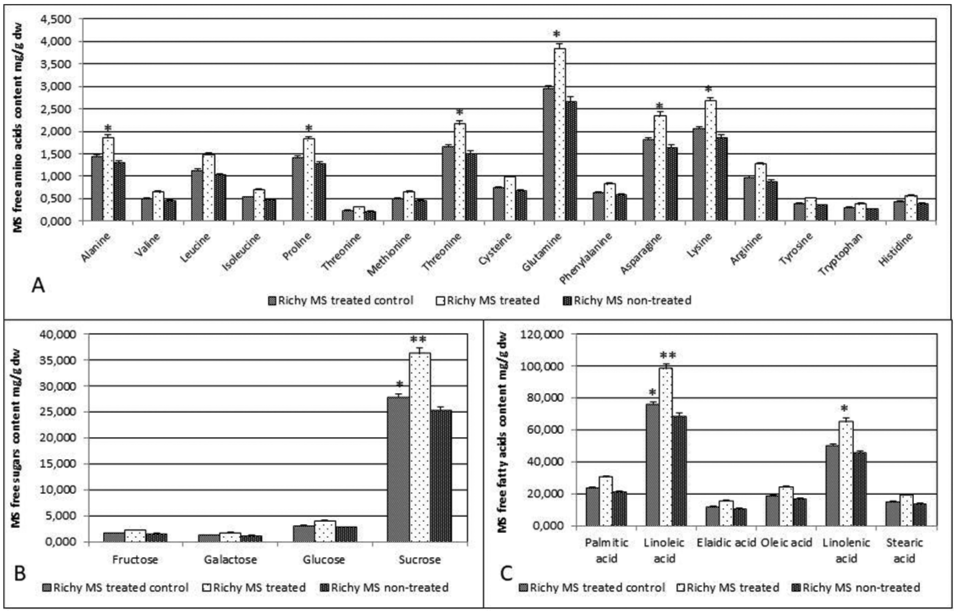

Fig. 9. Content of detected free metabolites (mg/g dw) in mature seeds (MS) in cv. Richy in 2021. A – content of free AAs; B – content of free sugars; C – content of free FAs. Data represent the mean ± SD. Asterisks denote statistically significant differences *p < 0.05; p < 0.01**

gen available to the plant tissues. Moreover they are not only a building units for proteins but also essential precursors or signaling molecule of biosynthesis of pigments, growth factors, lignin, nucleic acids, phytohormones, and phytoalexins (Bush, 1999). The elevated content of free AAs, sugars and fatty acids in MS contributed to the quality of soybean seeds. The performed approach of long lasting low temperature seeds pretreatment benefit seeds quality even in the years with not favorable environmental conditions.

Conclusion

The performed three-year experiment on investigated approach of long-lasting low temperature pretreatment of soybean seeds before sowing clearly indicated improved field performance and increased climate plasticity of plants grown from pretreated seeds. The metabolite profiles established during three consecutive years of free amino acids, sugars and fatty acids in soybean grains (green and mature) was strongly affected by the applied approach and was significantly elevated in pretreated seeds compared to 'non-treated'. The expression profiles obtained of selected AAs transporters clear demonstrated a correlation between the expression profiles and the content of AAs. The level of expression of the selected gene *Invertase* could serve as a marker for the level of free sucrose. The transcript profile of selected inorganic pyrophosphatase *H-PPase* could be applied as a marker for the plasticity of plants grown in the conditions of drought during the period of seeds filling. Altogether, the collected three years results confirm the benefits of low temperature pretreatment of seeds as a cheap approach, which improve plants field growth and the quality of seeds. Our future intents are focused on clarification of fine details of low-temperature pretreatment of seeds in respect to preserve the germination capacity in order to perform large-scale field experiment. The obtained results in the present study could benefit plant breeders, farmers and processors of soybean. The findings from this study contribute to the production of main legume crop by linking the sources and users of final food products.

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Text electronic supplementary material

Table S1. Sequences of primers used for qRT-PCR

Fig. S 1. Relative transcript level of *H-PP-ase* gene in leaves of plants grown from 'treated', 'treated control' and 'non-treated' seeds of the three investigated cultivars in 2021. Data represent the mean ± SE. The One-way ANOVA (Holm-Sidak) statistical test was applied to estimate the difference between all the variants. Asterisk denote statistically significant differences $*p < 0.05$;$** p < 0.01$; $*** p < 0.001$

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