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PHYSIOLOGICAL RESPONSES OF SUNFLOWER CLEARFIELD HYBRIDS TO THE HERBICIDE IMAZAMOX

Summary of doctoral dissertation

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The doctoral dissertation contains 141 pages, 6 tables and 18 figures, divided into 8 chapters. The used references are 199.

The official defense of the doctoral thesis is going to take place on 14 December, from 12.00 h, at 1a auditorium (Faculty of Agronomy).

Резюме

Слънчогледът е маслодайна култура, използвана основно като хранителен продукт, поради което получаването на високи добиви с ценни качества на продукцията са изключително важни за нарастващото население на Земята. Основен проблем за успешното отглеждане на слънчогледа е борбата с плевелите, които водят до значителни стопански загуби поради конкурентни отношения за основни хранителни ресурси. Приложението на хербициди е най-разпространеният метод за борба с плевелите при слънчогледа, но наред с него непрекъснато се търсят нови и по-ефективни средства за решаване на този проблем.

Системата Clearfield® предлага на земеделските производители ново и ефикасно решение за проблема с плевелите, комбинирайки високо селективни хербициди от групата имидазолините с имидазолин-устойчиви земеделски култури. Имазамоксът е един от петте имидазолинови хербицида, които блокират синтеза на аминокиселините с разклонена верига, инхибирайки активността на ензима ацетохидроксиацид синтаза (AHAS). Въпреки сравнително високата му селективност, често при обработка с имазамокс се наблюдава временно пожълтяване на вегетационния връх на слънчогледовите растения. Този хербициден стрес се усилва при предозиране или съчетаване на хербицидната обработка с неподходящи климатични условия. Механизмът на действие на имазамокса е добре известен, но последиците от инхибицията на ензима AHAS и нарушения синтез на аминокиселините левцин, валин и изолевцин върху основни физиологични процеси в растенията не са напълно изяснени. Известно е, че хербицидната детоксификацията при земеделските култури играе важна роля за тяхната селективност, но информацията за метаболизирането на хербицида имазамокс при слънчогледа е много ограничена. Лимитирана е и информацията относно протекторни ефекти на различни биостимуланти върху култури, третирани с имидазолинови хербициди, включително слънчоглед. Имайки в предвид тези непълноти в биологичните аспекти на системата, ние поставихме за цели на настоящата дисертация проучване на (1) физиологичната реакция на слънчогледови Clearfield хибриди към хербицида имазамокс и (2) възможности за подобряване на физиологичния статус на третираните растения чрез листно приложение на биостимуланти от групата на протеиновите хидролизати.

Изследванията, които са включени в настоящата дисертация, са проведени при няколко опитни постановки, а основните резултати са поместени в пет отделни части.

Толерантността на няколко слънчогледови Clearfield хибрида към хербицида имазамокс беше оценена чрез измерване модулацията на фотосинтезата на растения, третирани с препоръчителна и двукратно завишена доза имазамокс. Въз основа на измерените фотосинтетични параметри бяха установени различни нива на генотипна толерантност към хербицида имазамокс, като хибридът Милдими беше избран за най-толерантен и като такъв беше използван в следващите анализи (**Глава 3**).

Толерантността на растенията към различни стресови фактори, включително към третиране с хербициди, зависи и от така наречените специфични и неспецифични защитни механизми. За да се определи до каква степен и двата вида механизми се активират от хербицида имазамокс при слънчогледови растения, ние сравнихме отговорите на един толерантен и един устойчив към имазамокс слънчогледови хибриди след 24 часа третирането с хербицида. Получените резултати показаха различия в бързия стресов отговор на двата изследвани хибрида. При чувствителния сорт слънчоглед се задействаха предимно неспецифични механизми, като например ензимите от антиокислителната защитна система на клетката, докато при растения от толерантния хибрид бяха активирани механизмите за детоксификация и метаболизиране на ксенобиотици (**Глава 5**).

В дисертацията беше направено и мониторингово проучване на физиологичните ефекти от хербицида имазамокс във фазите на стрес и възстановяване (7 и 14 дни след третрирането) при имидазолин-резистентния слънчогледов хибрид Милдими. Периодите на стрес и възстановяване бяха определени на базата на линейният растеж на третираните с имазамокс слънчогледови растения (**Глава 5**). Нашите данни показват, че приложението на имазамокс причинява временно потискане на фотосинтезата, като това негативно въздействие беше установено както при светлинните, така и при тъмнинните реакции на фотосинтезата (**Глави 4, 5 и 6**). Анализът на растежа на третирани с имазамокс растения показва, че инхибирането на растежа е най-силно изразено 7 дни след третирането, а 14 дни след това растенията възстановяват своя растеж. В допълнение, бяха определени специфичната активност на ензима AHAS, експресията на гена *AHAS1* и остатъчните количества от хербицида имазамокс, като данните са подробн представени в **Глава 4**.

Относно влиянието на допълнително добавените амино киселини с разклонена верига върху физиологичният статус на слънчогледовите растенията третирани с имазамокс, в изследването се установи, че тяхното добавяне значително намалява негативния ефект от хербицида (**Глави 4 и 5**). В допълнение, третирането с

аминокиселини влияе положително върху ефективността на фотосинтезата чрез намаляване инхибицията на листния газообмен и на фотосинтетичния електронен транспорт, както и поради увеличаване съдържанието на фотосинтетични пигменти (**Глава 4**).

В дисертацията са включени резултати относно въздействието на листно приложен биостимулант (аминокиселинен екстракт) върху третирани с имазамокс имидазолинон-толерантни слънчогледови растения. Беше проведен OJIP тест за да се проучи в детайли ефекта на двата компонента върху фотосинтетичния апарат на третираните растенията. Като се имат в предвид получените резултати, може да се направи заключение, че комбинираното прилагане на имазамокс и биостимулант намалява негативните ефекти от хербицида, оказвайки положителен ефект върху светлинно-зависимите процеси на фотосинтеза (**Глава 6**).

Детайлна информация относно механизма на инхибиране на ензима AHAS, системата Clearfield и толерантността на земеделските култури към хербициди е поместена е **глава 1** (Introduction) от дисертационният труд. В **глава 2** (Materials and Methods) подробно са описани използваните в изследването материали, начини на приложение и аналитични методи. В заключителната **глава 7** (General Discussion and Perspectives) са обобщени основните резултати и заключенията от дисертационният труд. Цитираната научна литература е поместена в **глава 8** (References).

List of abbreviations

A – Photosynthetic rate (Net CO_2 assimilation)

AAE – amino acid extract

a.i. – Active ingredient

AHAS – Acetohydroxyacid synthase

BCAA – Branched chain amino acids

car – Carotenoids

CDNB – 1-chloro-2,4-dinitrobenzene

chl - Chlorophyll

ChlF - Chlorophyll fluorescence

ci – Intracellular CO_2 concentration

DAT – Day after treatment

DTE - Dithioerythritol

DTNB – 5,5′-Dithiobis(2-nitrobenzoic acid)

DW – Dryweight

E – Transpiration rate

EDTA - Ethylenediaminetetraacetic acid

ETR – Electron transport

eV - Electron volt

F – Fluorescence intensity

F_0 – Minimal fluorescence (dark adapted leaves)

FAD - Flavin adenine dinucleotide

F_J - Fluorescence intensity at the J-step (2 ms)

Fm – Maximal fluorescence (dark-adapted leaves)

Fm' - Maximal fluorescence (light-adapted leaves)

Fv/Fm - Maximal quantum yield of PSII

FW – Fresh weight

GPOD - Guaiacol peroxidase

GPX - Glutathione peroxidases

GR –Glutathione reductase

gs – Stomatal conductance

GSH - Glutathione

GSSG - Oxidized glutathione

GSTs - Glutathione S-transferases

IMI – Imidazolinones herbicides

IMI-R – Imidazolinine-resistance

LA – Leaf area

LC-MS - Liquid chromatography–mass spectrometry

m/z - Mass-to-charge ratio

NADPH - Nicotinamide adenine dinucleotide phosphate

PAR - Photosynthetic active radiation

POD – Peroxidases

PPFD - Photosynthetic photon flux density

PSII – Photosystem II

PVP - Polyvinylpyrrilidone

qN - Photochemical quenching

qP – Non-photochemical quenching

RGR – Relative growth rate

ROS - Reactive oxygen species

SOD – Superoxide dismutase

SPOD – Syringaldazine peroxidase

TBA - Thiobarbituric acid

TBArm - Thiobarbituric acid-reactive compound

TCA - Trichloroacetic acid

TPP - Thiamine pyrophosphate

Vt - Variable fluorescence

XOD - Xanthine oxidase

Y – Effective quantum yield of PSII

2VP – 2-vinyl-pyridine

1. INTRODUCTION, OBJECTIVES AND TASKS

Sunflower is one of the most important oil crops in the world, because of its good taste and healthy benefits (Dorrell and Vick, 1997). Unlike the other vegetable oils, about 90% of sunflower oil is used for human consumption and only 10% of total production is used for biodiesel and industrial application. Eastern Europe and the Black Sea Region represent more than 60% of the sunflower planted areas in the world (Kaya, 2014). At present in Bulgaria on average 500-600 thousand hectares of sunflower are planted and, therefore, the sunflower is the second most important crop after wheat. Sunflower is the main oilseed crop for Bulgaria, occupying about 85% of the area of industrial crops (Kostova, 2010).

Despite the high popularity of sunflower among the farmers and advanced technologies in its cultivation some diseases, insects and weeds can cause troublesome and costly problem. A study conducted for the National Sunflower Association of USA suggests that direct losses to world sunflower producers from weed competition, plant diseases and insect infestations during the mid-'90s ran in the area of $ 1.36 billion annually (https://www.sunflowernsa.com).

Weeds are one of the most limiting factors for global sunflower production causing considerable yield losses that are estimated at about 20-70 % (Blamey et al., 1997). Sunflowers are usually planted in low densities and grow slowly during the first weeks after planting until canopy closure. For that reason it is a poor competitor and weeds compete it successfully during these early growth stages and as most field crops, it is vulnerable to weed interference, during first 3 to 4 weeks after planting (Thompson et al., 2009). Maximum seed yields are reported when sunflower is kept weed free 4 to 6 weeks after planting (Reddy et al., 2015). In later phases of growing sunflower is a good competitor with weeds, but the greatest damage and yields losses of sunflower production are caused namely by weeds that emerge and establish in young stages of sunflower cultivation. Weeds which germinate before herbicide activation will not be controlled by a pre-emergence treatment. Herbicides are the most desirable method for weed control, especially under no-tilling conditions. Available post-emergence sunflower herbicides are mainly graminicides for grass control, but they do not control broadleaf weeds, which are another major problem with weed control in sunflower cultivation. Most of the herbicides commonly used in sunflower like Dinitroanilines and Chloroacetamides are primarily controlling grass weeds with narrow broadleaf weed control spectrum, especially on difficult- to-control weeds like *Xanthium strumarium, Datura stramonium, Ambrosia artemisiifolia*, and *Sonchu*s spp. Weeds insufficiently controlled in sunflower also include parasitic weeds like *Orobanche* spp (Pfenning et al., 2008). Therefore

weed management is an important component of successful sunflower cultivation and production.

The Clearfield® production system is a new tool for growers to optimize production in many crops, including sunflower. It presents agricultural system combining imidazolinone-tolerant crops with imidazolinone herbicides. Imazamox is high selective imidazolinone herbicide, which suppressing the branched chain amino acids biosynthesis in plants by inhibiting the enzyme activity of the kay enzyme acetohydroxyacid synthase (AHAS). The mode of action of imazamox is well known, but the exact consequences of AHAS inhibition in the cardinal physiological processes in plants, remain not unclear. The most common inhibitory effect of AHAS herbicides is the growth arrest of meristem tissues of non-target plants. Although the high selectivity of IMI-R sunflower hybrids, transitions crop injury occasionally due to higher imazamox rates, application timing as well as the environmental conditions.

It is known that the herbicide degradation in crops plays a major role in herbicide selectivity and the enzyme family such as Cytochrome P450 and Glycosyltransferase are involved in herbicide degradation, but the information concerning the role of glutathione S-transferases is still incomplete. In addition, there is a luck of information about the possible protective effects of different groups of biostimulants on imidazolinone-treated crops, including sunflower. Concerning these ambiguities in current study we aimed (**I**) to reveal the degradation rate and physiological effects of the herbicide imazamox in IMI-resistant and IMI-susceptible sunflower hybrids and (**II**) to examine the some possibility of reducing the temporary growth inhibition caused on herbicide imazamox on sunflower plants by foliar application of amino acid extract.

To achieve the objectives of our research we set the following tasks:

1) to evaluate the tolerance of sunflower Clearfield hybrids to both recommendable and higher doses of the herbicide imazamox.

2) to compare the fast (24h) response of IMI-R and IMI-S sunflower hybrids to the herbicide imazamox.

3) to monitor the physiology alterations of imazamox-treated IMI-R sunflower hybrids in stress and recovery phases.

4) to estimate the influence of BCAA addition in the root medium on physiological performance of imazamox-treated sunflower plants.

5) to evaluate the effect of leaf applied biostimulant (amino acid extract) on imazamox-treated IMI-tolerant sunflower plants.

2. MATERIALS AND METHODS

2.1. Plant material

In this work 6 sunflower hybrids were used:

- 5 commercial Clearfield hybrids: Alego, LG 56.58, Primis, Mildimi and Tektonik.
- 1 conventional hybrid - Albena

The main part of the research were conducted with hybrids:

- ✓ Mildimi - who was selected after comparative study between the above mentioned CL hybrids. Mildimi (IMI-R) carrying the haplotype 5 of the AHAS1 gene (*Imisun* trait). The CL hybrid Mildimi is Produced by Syngenta. Main characteristics: fairly late hybrid, fairly high plant, potential fat content – 45%, tolerance to Sunflower Broomrape (*Orobanche cumana*): A-E. The hybrid is produced by Syngenta.
- ✓ Albena - the hybrid has been developed by the Dobrudzha Agricultural Institute (General Toshevo, Bulgaria). Standard (linoleic) type of oil. High yield hybrid resistant to Sunflower Broomrape (*Orobanche cumana*): A-E and sunflower downy mildew (*Plasmopara halstedii*).

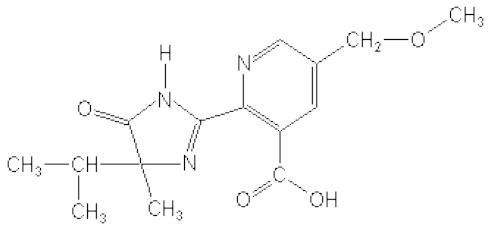
2.1.Chemicals used in the study

2.1.1. Imazamox /Pulsar 40/

Imazamox –

2-(4-Isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl)-5-methoxymethylnicotinic acid

Empirical formula: $C_{15}H_{19}N_3O_4$, Molecular weight: 305.32906 g/mol



LD_{50} value (rat oral) – 5000 mg/kg (Directive 401 of OEDC, Organization for Economic Cooperation and Development)

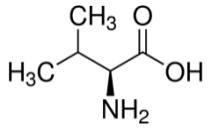
The commercial herbicide product used in this study is Pulsar 40, produced for the eastern Europe countries (Bulgaria, Croatia, France, Greece, Hungary, Italy, Romania Serbia, Slovakia, Spain, Turkey), by BASF chemical company. The Pulsar 40 is suspension concentrate with active ingredient imazamox in concentration 40 g.L-1. Pulsar 40 is categorized as selective, contact, soil and vegetation imidazolinone herbicide. Quarantine period – 90 days.

2.2.2. Branched chain amino acids (BCAA)

2.2.2.1. L-Veline – ((S)-α-Aminoisovaleric acid, L-2-Amino-3-methylbutanoic acid), powder form.

Empirical formula: $(CH_3)_2CHCH(NH_2)CO_2H$, Molecular weight: 117.15 g/mol.

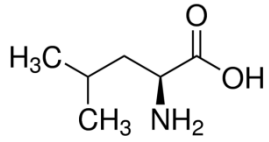
Reagent grade ≥ 98% (HPLC), produced by Sigma Aldrich (V0500, CAS Number 72-18-4).



2.2.2.2. L-Leucine – ((S)-2-Amino-4-methylpentanoic acid), powder form.

Empirical formula: $(CH_3)_2CHCH_2CH(NH_2)CO_2H$; Molecular weight: 131.17 g/mol.

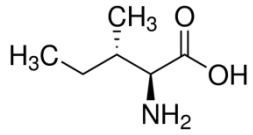
Reagent grade ≥ 98% (HPLC) produced by Sigma Aldrich (L8000, CAS Number 61-90-5)



2.2.2.3. L-Isoleucine – ((2S,3S)-2-Amino-3-methylpentanoic acid), powder form.

Empirical Formula: $C_2H_5CH(CH_3)CH(NH_2)CO_2H$, Molecular weight: 131.17 g/mol.

Reagent grade ≥ 98% (HPLC) produced by Sigma Aldrich (I2752, CAS Number 73-32-5)



2.2.3. Terra-Sorb foliar

Terra-sorb foliar is biostimulant with an amino-acid base obtained by enzymatic hydrolysis containing. The product is used to prevent situations that cause crop stress and incense of flowering and fruit setting in critical conditions. Terra-sorb foliar is recommended

for field crops, fruits and vegetables. The formula contains all biologically active free amino acids: Asp, Ser, Glu, Gly, His, Arg, Thr, Ala, Pro, Cis, Tyr, Val, Met, Lys, Ile, Leu, Phe, Trp) - 9.3% (w/w), Total nitrogen (N) - 2.1% (w/w), Organic nitrogen (N) - 2.1% (w/w), Boron (B) - 0.02% (w/w), Manganese (Mn) - 0.04% (w/w), Zinc (Zn) - 0.07% (w/w), Organic matter - 14.8% (w/w). Terra-sorb foliar is produced by Bioiberica S.A. (Spain).

2.3. Methods

2.3.1. Determination of glutathione content

Concentrations of reduced (GSH) and oxidized (GSSG) glutathione were determined according to the method described by Queval and Noctor (2007). Frozen leaf tissue was ground, homogenized in HCl and centrifuged. NaH_2PO_4 buffer was added to the supernatant and pH was adjusted to 4.5. Estimations of GSH and GSSG are based on the glutathione reductase (GR)-dependent reduction of 5,5-dithiobis(2-nitro-benzoic acid), monitored at 412 nm. To determine total glutathione, the supernatant was added in triplicate to a 96-well plate. The reaction was started by adding GR (20U/ml) and the rate of DTNB reduction was monitored. Sample concentrations of total glutathione were calculated relative to a GSH standard curve, and were corrected for GSH-independent reduction of DTNB by subtraction of the mean value of duplicate blank assays (0 nmol GSH). GSSG was determined according to the same principle after incubation with 2-VP to complex GSH. The final standard was also subjected to incubation with 2- VP. Concentrations of GSSG were calculated as for total glutathione.

2.3.2. Detemination of AHAS enzyme activity

The protein extraction was performed according to Schröder and Götzberger (1997). Frozen plant material (0.5 g) was powdered and 5 ml of freshly prepared extraction buffer [0.1 M Tris/HCl, pH 7.8, 5 mM EDTA, 5 mM dithioerythritol, 1% Nonidet P40, 1% insoluble polyvinylpyrrolidone (PVP)] was added, homogenized, and extracted for 30 min before centrifugation for 30 min at 20,000 rpm. The proteins in this crude extract were precipitated by addition of ammonium sulfate in two steps of 40 and 80% saturation, respectively. Protein solutions were centrifuged after each step and pellets finally resuspended in 2 ml of 25 mM Tris/HCl buffer (pH 7.8). This step was followed by desalting with Sephadex PD-10 columns.

Acetohydroxyacid synthase (AHAS, EC 2.2.1.6) enzyme activity was measured according to Ray (1984) with some modifications. This assay detects acetolactate, the AHAS enzyme product of, after conversion to acetoin.

Leave tissue (2g) was cold ground in 6 ml extraction buffer (0.1 M potassium phosphate (pH 7.5) containing sodium pyruvate (0.1 M), thiamine pyrophosphate (0.5 mM), $MgCl_2$ (0.5 mM), flavin adenine dinukleotide (FAD, 10 µM) and 10% glycerol) and PVP were added. The homogenates were filtered through a layer of cheese cloth and centrifuged at 14 000g for 20 min at 4°C. Proteins were precipitated from the supernatant at 50% of saturation of ammonium sulfate and solution centrifuged at 14 000g for 25 min at 4°C. The supernatant was discarded and pellets were resuspended in potassium phosphate (0.1 M, pH 7.5) containing sodium pyruvate (20 mM), and $MgCl_2$ (0.5 mM).

AHAS activity was assayed by adding 0.1 ml of enzyme extract to 0.5 mL reaction mixture (20 mM potassium phosphate (pH 7.5) containing sodium pyruvate (20 mM), thiamine pyrophosphate (0.5 mM), $MgCl_2$ (0.5 mM) and FAD (10 µM)). The reaction mixture was incubated in dark at 37 °C for 1 h and the reaction was subsequently terminated by adding 50 µL H_2SO_4 (3M). Then 0.5 mL 0.5% creatine was added and the solution was incubated in dark at 60 °C for 15 min. The added sulfuric acid terminated the AHAS reaction and decarboxylated the enzyme product acetolactate to acetoin. Acetoin was detected as a colored complex (A = 525 nm) formed after adding 5% α-naphthol (freshly prepared in 2.5 M NaOH) and fallowed by dark incubation at 60 °C for 15 min. A standard curve was constructed using commercial acetoin.

2.3.3. Gene expression analysis

RNA was extracted from disrupted tissue using the miRVANA Total RNA Isolation kit, according to the manufacturer's instructions (Life Technologies). To remove genomic DNA, 1 µg RNA was treated using the TURBO DNA-free kit according to the manufacturer's instructions (Life Technologies). First strand cDNA synthesis was performed with 1 µg total RNA using the High Capacity reverse transcription kit and a combination of oligo(dT)-primers and random hexamers according to the manufacturer`s instructions (Life Technologies). The cDNA sample was diluted 10-fold in 1/10 TE-buffer and stored at -20°C. Quantitative PCR was performed with the 7500 Fast real-time PCR cycler (Applied Biosystems) and SYBR green chemistry. PCR reactions were carried out in a total volume of 10 µl, containing 2 µl cDNA sample, 5 µl Fast SYBR green Master Mix (Life Technologies) and 300 nM of each primer. Primer sequences of reference genes were according to Fernandez et al. (2011) Sequences of the GSH1 and GSH2 genes were searched through Gene Index (http://compbio.dfci.harvard.edu/cgi-bin/tgi/geneprod_search.pl). Specificity of the primers was checked by BLAST and melting curve analysis after real-time PCR.

The amplification efficiencies of all primer sets were investigated by measuring a 2-fold serial dilution (E=10(-1/slope) method) and were approved when they were greater than 1.8. Genes and primer sequences for RT-qPCR are listed in supplemental table. After geNorm analysis (Vandesompele et al., 2002), two reference genes (ACT and PEP), were selected for normalization.

Relative quantities were calculated as 2-ΔCq and normalized relative quantities by dividing relative quantities by the normalization factor based on the geometric mean of the expression level of two reference genes. Adherence of the RT-qPCR to MIQE guidelines (Bustin et al., 2010) is also listed in supplemental table.

2.3.4. Chlorophyll *a* fluorescence analysis

Analysis carried out with MINI-PAM

Chlorophyll fluorescence measurements were performed on intact, dark- and light-adapted leaves with a pulse modulation fluorometer (MINI-PAM, Heinz Walz, Germany), before taking samples for biochemical assessment. Plants were kept in the dark for at least 25 min before measurement. By switching on the measuring beam (0.02 – 0.20 μmol m-2 s-1), the minimal level of fluorescence (F_0) was recorded. Immediately thereafter, a saturating light pulse (SLP) of 5500 μmol m-2 s-1 with 0.8 s duration was sent out to record the maximal level of fluorescence in the dark-adapted state (Fm), from which the maximal quantum yield of PSII [Fv/Fm] was calculated (with Fv = Fm – F0). After 30 min of light adaptation at 250 μmol m-2 s-1, the steady-state level of photosynthesis was reached and a saturating light pulse (5500 μmol m-2 s-1) was applied. Based on the measurements of fluorescence yield before the SLP (F) and the maximal fluorescence reached during the SLP (Fm'), following parameters could be calculated: the effective yield of photochemical energy conversion [Y = (Fm' – F) / Fm'] and the electron transport rate [ETR = Y*PAR*0.5*0.84] (White and Critchley, 1999). According to Schreiber (2004), photochemical quenching [qP = (Fm' – F) / (Fm' – F0)] and non-photochemical quenching [qN = (Fm – Fm') / (Fm – F0)] quenching parameters, as well as NPQ [(Fm – Fm') / Fm] were calculated.

Analysis carried out with Handy PEA

After 30 min light adaptation at 250 μmol m-2 s-1 steady-state level of photosynthesis was achieved and a saturating pulse with the same characteristics was applied. Fluorescence yield before triggering of saturation pulse (F), maximal (Fm') fluorescence reached during the saturation pulse, effective yield of photochemical energy conversion (Y), Y = (Fm'-F)/Fm') as

well as apparent electron transport rate (ETR) calculated as ETR = Y*PAR*0.5*0.84 (White and Critchley, 1999) were determined.

Very sensitive method, which allows evaluating the change in the general bioenergetics status of plants, particularly Photosystem II (PSII) status and linear electron transport rate, is measurement of chlorophyll *a* fluorescence (ChlF) (Papageorgiou and Govindjee 2004). The method is based on high time-resolution measurements of the fast photoinduced changes of chlorophyll *a* fluorescence emitted mainly by antennae pigments of PSII. The kinetics of fast ChlF rise after illumination of dark adapted photosynthesizing samples show some specific phases denoted with letters OJIP. The shape of the ChlF rise reflects the electron transport events in photosynthetic electron transport chain starting from water oxidation reactions in PSII and ended by NADP reduction by photosystem I (PSI).

The important structural and functional characteristics of thylakoid membranes could be described by a number of parameters calculated from ChlF rise data and called OJIP test. The fluorescence intensities determined at 50 μs, 100 μs, 300 μs, 2 ms, 30 ms and F_M were used for the calculation of the OJIP test parameters (Strasser and Srivastava et al. 1995).

Induction curves of ChlF for 1 s with 3000 $\mu mol.m^{-2}.s^{-1}$ PPFD after 1 hour dark-adaptation were recorded in native leaves of *Helianthus annuus* Clearfield® by fluorimeter Handy PEA (Handy Plant Efficiency Analyzer) Hansatech Instruments Ltd., King's Lynn, UK for 1 s. The measured spots of the leaves were dark-adapted for 1 hour while the whole plants were left on light. For each experimental variant at least 10 measurements were done. The primary data processing was done by program HandyBarley, developed by Petko Chernev at the Department of Biophysics and Radiobiology, Faculty of Biology, Sofia University, and the secondary processing, including calculation of JIP parameters – on Microsoft Excel. The plots were done in Sigma Plot.

2.3.5. Statistical analysis

Statistical analysis was performed using ANOVA (for $P< 0.05$). Two way ANOVA was used to compare the responses of both hybrids to the treatments only at 1 DAT. Thereafter (7 and 14 DAT) the data within each time point were analyzed using one way ANOVA. Based on ANOVA results, a Duncan test for mean comparison was performed, for a 95% confidence level, to test for significant differences among treatments. In the figures, different letters (a, b, c) express significant differences.

3. RESULTS

3.1. Tolerance of IMI-R sunflower hybrids to the herbicide imazamox

Experimental design

Pot-soil experiments were carried out in the greenhouse. The sunflower plants were grown in pots filled with approximately 6-7 cm drainage (boulders with size 1 – 1,5 cm) and 5 kg dry soil taken from the experimental field of the Agricultural University of Plovdiv. During the vegetation the watering was performed by a plastic pipe directly in the drainage layer up to 60-70% soil moisture every two days. Fertilization with 500ml ½ Hoagland solutions was done once, two weeks after sowing.

When plants reached the 4-6 leaf stage, a two-factorial experimental design was set up: first factor – genotype (the 5 hybrids given above) and second factor – herbicide imazamox treatment (non-treated control, treated with the recommended field imazamox dose (4.8 g a.i. / da = 120 ml Pulsar 40 / da) and the double imazamox dose (9.6 g a.i. / da = 240 ml/da Pulsar 40). Each of the variants was replicated in three pots, 4 plants per pot.

Data presented in Table 1 show that treatment of sunflower plants with imazamox in the recommended dose (4.8 g / da) resulted in a significant decreases of the net photosynthetic rate (A) ($P < 0.05$), except for cv. Mildimi. The inhibition of A varied between 33 and 41%. As expected, the decrease of A was higher in plants treated by the double imazamox dose. Our results confirm the opinion (Pfenning *et al.,* 2008) that the imazamox can cause a slight negative impact on tolerant plants even when it is applied in the recommended dose. The results obtained in our study correspond also with those of Gaston *et al.* (2002) who showed an inhibition of A in imazethapyr treated pea plants. Our results provide evidence that the sunflower hybrid Mildimi showed better tolerance to imazamox treatment compared to the other cultivars. The net photosynthetic rate in imazamox-treated plants from this cultivar was almost not affected by both the recommended and the double imazamox dose.

The net photosynthetic rate represents the state of the overall photosynthetic process – CO_2 assimilation. In fact, the photosynthetic process involves reactions at different functional levels – pigment level, primary light reactions, thylakoid electron transport reactions, dark-enzyme stromal reactions as well as slow regulatory feedback processes. The herbicide treatment can influences directly or indirectly any of these processes. Chlorophyll fluorescence is another possible approach for indirect judgment of plant photosynthetic performance. This arises from the fact that fluorescence is complementary to the alternative

pathways of utilization of absorbed sunlight energy, which are photochemistry (photosynthesis) and heat dissipation. Briefly, the fluorescence yield is the highest when the photochemistry and heat dissipation are the lowest.

The results presented in Table 1 show also that Y of herbicide-treated sunflower plants were diminished in the studied cultivars, but to different degrees – from 9 to 26% in the recommended dose and similarly in the double one. The tendency was significant in cultivars. Tektonik and Alego treated with the recommended dose and cultivars Tektonic and Primis treated with the higher imazamox dose. It should be mentioned also that the decrease of Y was obviously smaller than that of A, which indicates that the primary light reactions are less affected than other photosynthetic processes. The results obtained in our study correspond with the findings of Sousa *et al.* (2013) who reported that the imidazolinone herbicides can cause inhibition of photosynthetic electron transport reactions.

Table 1: *Net photosynthetic rate (A) and quantum yield of PSII photochemistry (Y) of plants from different Clearfield sunflower hybrids 7 days after treatment with the herbicide imazamox. The values represent the mean of three biological replicates. Different letters (a, b, c) express significant differences (P < 0.05) within the treatments of each cultivar.*

Cultivars / Treatments	Control	4,8 g/da imazamox	9,6 g/da imazamox
	A (μmol m^{-2} s^{-1}) (In parenthesis -% from control)		
LG 56.58	20,48 (100) a	11,99 (59) c	14,96 (73) b
Tektonik	19,20 (100) a	12,11 (63) b	10,40 (54) b
Alego	22,41 (100) a	14,10 63) b	10,67 (47) b
Mildimi	20,98 (100) a	20,39 (97) a	19,91 (95) a
Primis	18,68 (100) a	12,57 (67) b	9,49 (51) b
	Y (In parenthesis -% from control)		
LG 56.58	0,192 (100) a	0,172 (90) a	0,167 (87) a
Tektonik	0,254 (100) a	0,188 (74) b	0,194 (76) b
Alego	0,259 (100) a	0,219 (79) ab	0,194 (78) b
Mildimi	0,264 (100) a	0,239 (91) a	0,228 (86) a
Primis	0,213 (100) a	0,187 (88) a	0,137 (65) b

On the 14th DAT (Table 2) the values of A in both non-treated (control) and treated with the recommended imazamox dose (4.8 g a.i. / da) plants were similar, indicating significant recovery of the photosynthetic apparatus. The Y values showed the same tendency: the determined values were not significantly different from the control plants. Photosynthetic measurements of plants treated with the double imazamox dose were not performed on the 14th DAT due to visual damages of the respective leaves, such as necrotic spots or occurrence

of both chlorosis and epinasty symptoms. Nevertheless, it is important to mention that the developing new leaves were without toxicity symptoms.

Table 2: *Net photosynthetic rate (A) and quantum yield of PSII photochemistry (Y) of plants from different Clearfield sunflower hybrids 14 days after treatment by the herbicide imazamox The values represent the mean of three biological replicates. Different letters (a, b, c) express significant differences (P < 0.05) within the treatments of each cultivar.*

Cultivars / Treatments	Control	4,8 g/da imazamox
	A (μmol m^{-2} s^{-1}) (In parenthesis -% from control)	
LG 56.58	17,82 (100) a	17,05 (96) a
Tektonik	14,80 (100) a	14,66 (99) a
Alego	15,99 (100) a	16,10 (101) a
Mildimi	16,91 (100) a	17,81 (105) a
Primis	17,22 (100) a	13,41 (78) b
	Y (In parenthesis -% from control)	
LG 56.58	0,218 (100) a	0,192 (88) a
Tektonik	0,281 (100) a	0,223 (79) a
Alego	0,216 (100) a	0,226 (104) a
Mildimi	0,239 (100) a	0,230 (96) a
Primis	0,207 (100) a	0,202 (98) a

The biometrical analyses of plants, performed on the 14th DAT, are presented in Figure 1. The results show that the growth of imazamox-treated plants from all sunflower Clearfield hybrids was inhibited, but to a different extent in-between cultivars.

The height of plants from cultivars LG56.58, Tektonik and Meldimi (Figure 1 B), treated with the recommended imazamox dose, was significantly reduced in comparison to non-treated (control) plants, while the plants from cultivars Alego and Primis were only slightly (not significantly) lower. The height of plants treated with the double imazamox dose was strongly diminished in all tested cultivars.

The treatment of sunflower plants with imazamox resulted in inhibition of fresh weight accumulation (Figure 1 A). The fresh weight of plants treated with the recommended dose of imazamox was significantly lower than the respective controls for cultivars LG56.58 and Alego and showed a decreasing trend in other cultivars. Again, the double dose imazamox strongly inhibited the growth from all cultivars.

The development of the leaf area in imazamox treated plants was also affected (Figure 1 C). The herbicide, applied in the double dose, caused inhibition (about 50% or higher) of

this parameter, while in the recommended dose a slight but significant inhibition was observed only in plants from cultivars LG56.58 and Primis.

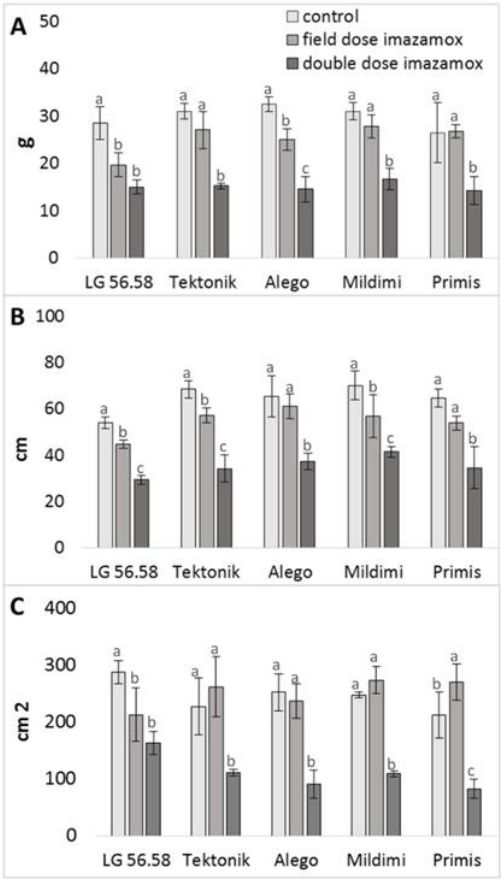


Figure 1: *Biometrical parameters: A – weight of plants; B – height of plants and C – leaf area, of Clearfield sunflower hybrids treated with field dose (4.8 g a. i./ha) and double dose (9.6 g a. i. /ha) of herbicide imazamox, 14 days after treatment. The values represent the mean of three biological replicates. Different letters (a, b, c) express significant differences (P < 0.05).*

Considering these results we concluded that the treatment of Clearfield sunflower hybrids with the recommended field dose imazamox (48 g a.i. ha^{-1}) causes a transient and recoverable inhibition of both plant growth and photosynthetic performance, while the doubled dose leads to significant damages and incomplete recovery. The IMI-R sunflower hybrids LG 56.58, Tektonic, Alego, Mildimi and Primis differ in their tolerance to the herbicide imazamox. Assessment of the physiological status of imazamox-treated plants assigns the highest tolerance to the hybrid Mildimi.

3.2. Fast response of IMI-R and IMI-S sunflower hybrids to the herbicide imazamox and physiology alterations of imazamox-treated IMI-R sunflower hybrids in stress and recovery phases

Experimental design

The experiments were carried out in climatic modules in a Fitotron chamber (H.V.A. – Koeling BVBA, Genk, Belgium) in the Hasselt University, Belgium. The seeds were rinsed in distilled water and placed for two hours in distilled water for imbibition. After that the seeds were placed in perlite for germination in 25 ^{o} C. When they reached the suitable size, they were betted on Styrofoam by sticky strip. The plants were placed in 2.5 liters pots (4 plants per pot) filled with ½ modified Hoagland nutrient solution.

The plants from two hybrids IMI-R Mildimi and IMI-S Albena were grown in a growth chamber with a 14/10 hours (light/dark) photoperiod, 250 μmol m^{-2} s^{-1} photosynthetic active radiation (PAR) at leaf level, temperature 24/22 ± 1 C° day/night and 60 % relative air humidity. The aeration was carried out by aquarium aeration system in each pot, night and day. At 4-6 thru leaf stage the plants were treated and were arranged an experimental design with four treatments:

1. Control – non-treated plants
2. BCAA – 0,5mM of each Branched chaun amino acids (Valine, Leucine and Isoleucine).
3. Imazamox – 40 g a.i. h^{-1} or approximately 132 μg per plant.
4. Imazamox + BCAA - 40 g a.i. h^{-1} or approximately 132 μg per plant + 0,5mM of each Branched chaun amino acids (Valine, Leucine and Isoleucine).

Each of the four variants was repeated in three pots (4 plants per pot).

Plant tolerance to herbicides at the biochemical level depends on both specific and non-specific defence mechanisms. Usually, quick plant responses to different stress factors are based on non-specific mechanisms. To identify to what extent both kinds of mechanisms are induced in sunflower plants by the herbicide imazamox we compared the responses of IMI-R (Mildimi) and IMI-S (Albena) sunflower hybrids 24 hours after the treatment.

IMI-S sunflower hybrid showed an increased GR activity (enzyme catalyzing the conversion of oxidized glutathione - GSSG to reduced glutathione - GSH) at 1 DAT after imazamox treatment (Fig. 2). Similarly, significantly increased enzyme activities were observed also in GPX and SPOD enzymes. In comparison, the activities of antioxidative enzymes in IMI-R plants treated with imazamox were not significantly higher at 1 DAT, where only the SPOD showed slightly increased activation. The activity of GR was different

in imazamox-treated IMI-R plants, compared to the one in the susceptible hybrid where at the first DAT there was no significance enzyme activation.

Glutathione is a key metabolite in plants preventing damage to cellular components caused by ROS (Noctor and Foyer, 1998) and is used also by the GSTs for the detoxification of toxic xenobiotics such as herbicides (Jozefczak et al., 2012). The total GSH contents and the percentages of GSH/GSSG in imazamox treated sunflower plants are presented in Figure 3A. At 1 DAT total GSH contents in IMI-R plants were similar for all treatments except for BCAA-treated plants in which a slight increase in GSH was observed. The plants from conventional hybrid showed increased GSH content in the variant treated with imazamox. No significant changes in the GSH/GSSG ratio were observed in both hybrids and all treatments.

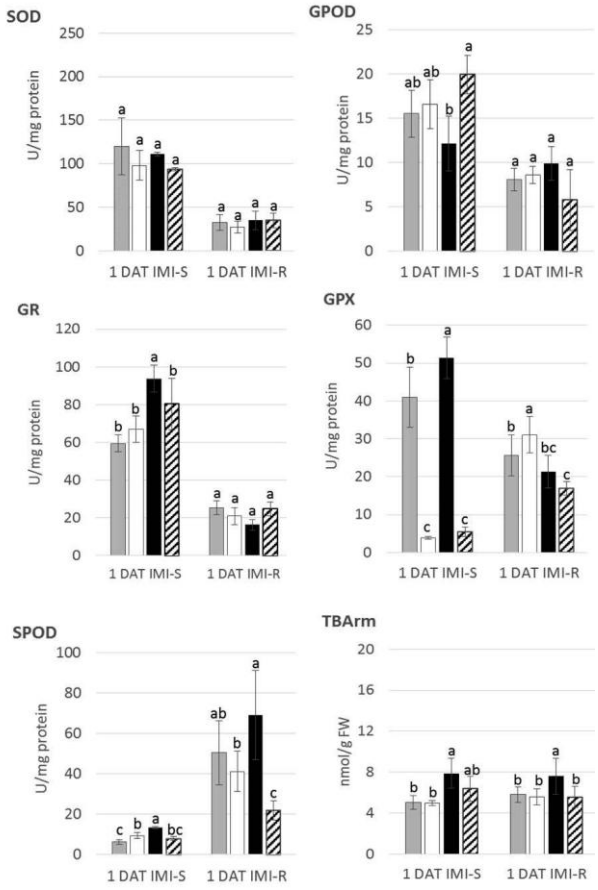


Figure 2: *Activities of anti-oxidative enzymes and level of lipid peroxidation determined as levels of the TBA-reactive compounds (TBArm) in leaves of IMI-S and IMI-R sunflower plants treated with the herbicide imazamox with or without supply of BCAA expressed in days after treatment (DAT). Different letters (a, b) express significant differences between treatments at each time point (P < 0.05). Significance levels: For SOD: treatment effect – NS, hybrid - *** P< 0.001, treatment*hybrid – NS; For GPOD: treatment effect – NS, hybrid - *** P < 0.001, treatment*hybrid – ** P< 0.01; For SPOD: treatment effect – ** P< 0.01, hybrid - *** P < 0.001, treatment*hybrid – ** P< 0.01; For GPX: treatment effect – *** P< 0.001, hybrid - NS, treatment*hybrid – *** P< 0.001; For GR: treatment effect – NS, hybrid - *** P < 0.001, treatment*hybrid – *** P< 0.001; For TBArm: treatment effect – *** P< 0.001, hybrid - NS, treatment*hybrid – NS.*

To estimate the impact of imazamox treatment on the fast xenobiotic metabolism of sunflower plants the activity of glutathione-S-transferase (GST) was determined with two substrates, more specifically CDNB and fluorodifen. While no increase in GST activity was observed in IMI-S sunflower plants, the activity with both substrates was higher after imazamox application in IMI-R plants (Fig. 3 B). In contrast to the IMI-S plants, the GST-fluorodifen activity was enhanced from the first day after imazamox treatment in the IMI-R hybrid. At the molecular level, the responses in the IMI-R and IMI-S hybrids were similar: imazamox treatment led to increased *GSH2* expression in both hybrids. The results regarding the expression of *GSH1* and *GSH2* genes supported the fact that imazamox could induce a GSH accumulation via increased expression of synthesis genes (Fig. 3 C).

Our results showed dissimilarities in the fast stress response and defence mechanisms in the compared hybrids. In the IMI-S sunflower hybrid Albena, the total amount of the metabolite glutathione was increased after imazamox application. These plants responded with activation of the antioxidative cell defense network manifested by enhanced antioxidant enzyme activities, such as GR, GROX and SPOD. In contrast, in IMI-R hybrids almost no changes in the antioxidative enzyme activities were observed, while GSTs activities were significantly higher.

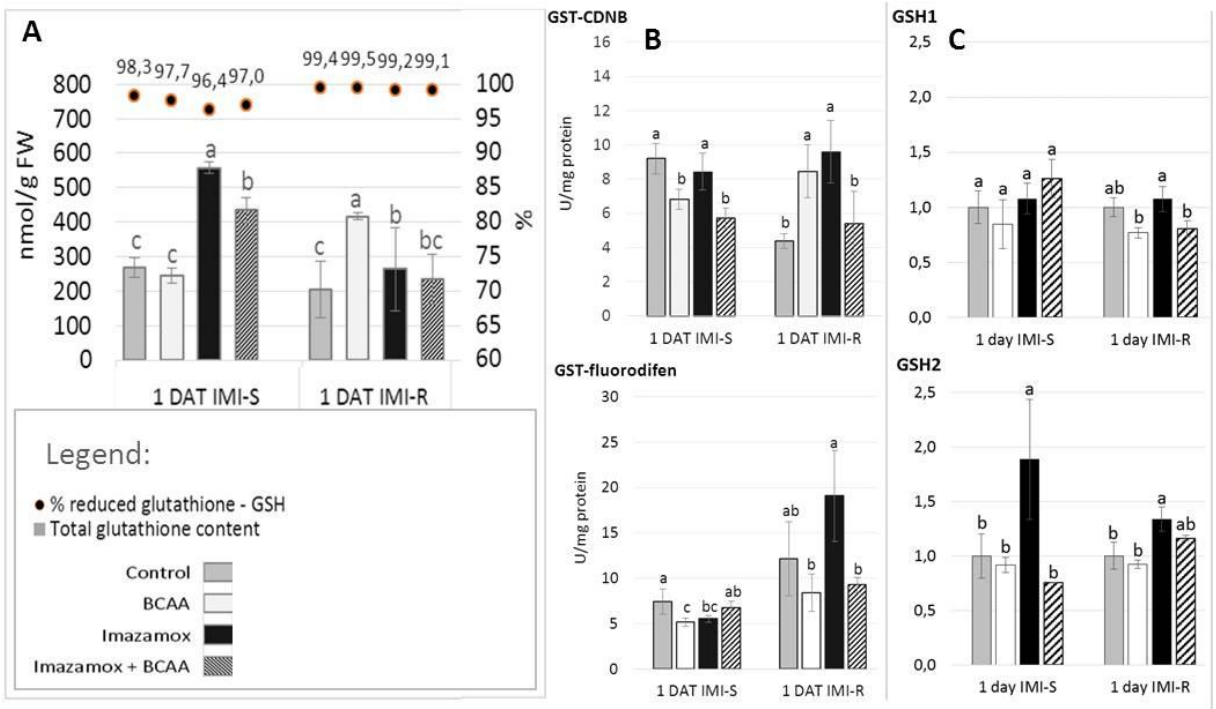
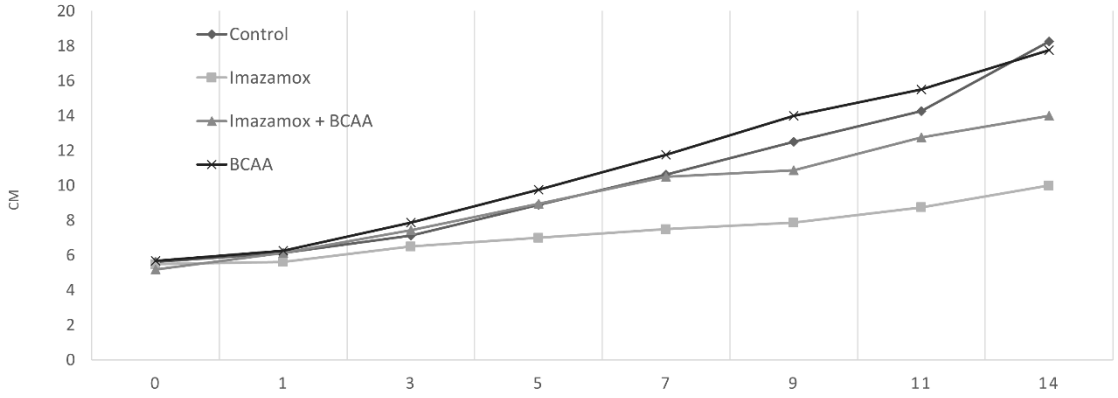


Figure 3: A *- Total glutathione contents (bars) and percentages of reduced glutathione (points) in IMI-S and IMI-R sunflower plants treated with the herbicide imazamox with or without supply of BCAA expressed in first day after treatment (DAT). B - Activity of glutathione S-transferases, substrate CDNB and fluorodifen, in leaves of IMI-S and IMI-R sunflower plants treated with the herbicide imazamox with or without supply of BCAA expressed in first day after treatment (DAT).* ***B - :*** *Relative expression: GSH1 gene, encoding the enzyme gamma-glutamylcysteine syntethase (EC 6.3.2.2.) and GSH2 gene, encoding the enzyme glutathione synthetase (EC 6.3.2.3.), in IMI-S and IMI-R sunflower plants treated with the herbicide imazamox with or without supply of BCAA expressed in first day after treatment (DAT).The values represent the mean of three biological replicates. Different letters (a, b) express significant differences between treatments at each time point (P < 0.05). Significance level at 1 DAT: For GSH content (treatment effect – *** P< 0.001, hybrid - *** P< 0.001, treatment*hybrid – *** P< 0.001); For CDNB (treatment effect – ** P< 0.01, hybrid - NS, treatment*hybrid – ** P< 0.01); For fluorodifen: (treatment effect – ** P < 0.01, hybrid - *** P < 0.001, treatment*hybrid – ** P< 0.01); For GSH1 (treatment effect – NS, hybrid - NS, treatment*hybrid – NS); For GSH2: (treatment effect – ** P < 0.01, hybrid - * P < 0.05, treatment*hybrid – NS).*

3.3. Monitoring of the physiology alterations of imazamox-treated IMI-R sunflower hybrids in stress and recovery phases.

The height of sunflower plants was monitored during 14 days after the start of the treatment. Height of the IMI-R hybrid is presented in figure 4. The growth retardation of imazamox-treated sunflower plants relative to non-treated plants started immediately and the absolute difference in height of the plants gradually increased to the end of experimental period. The growth of imazamox-treated sunflower started to recover from the 9th day after treatment (DAT), but the recovery was not sufficient to compensate the initial growth inhibition and at 14 DAT the non-treated plants were over 43 % taller than the imazamox-treated sunflower plants. At the same time point, the growth inhibition of plants receiving combined application of imazamox and BCAA was only 24% compared to the non-treated plants.



Control	5,6 ± 0,5 a	6,1 ± 0,7 ab	7,1 ± 0,8 b	8,9 ± 0,7 b	10,6 ± 1,2 a	12,5 ± 1,7 ab	14,3 ± 2,1 a	18,3 ± 2,9 a
BCAA	5,7 ± 0,5 a	6,3 ± 0,5 a	7,9 ± 0,5 a	9,8 ± 0,7 b	11,8 ± 1,2 a	14,0 ± 1,2 a	15,5 ± 0,6 a	17,8 ± 2,5 ab
Imazamox	5,5 ± 0,4 a	5,6 ± 0,4 b	6,5 ± 1,0 c	7,0 ± 1,0 c	7,5 ± 1,2 b	7,9 ± 1,4 c	8,8 ± 1,7 b	10,0 ± 2,7 c
Imazamox + BCAA	5,2 ± 0,5 a	6,1 ± 0,2 ab	7,4 ± 0,3 ab	8,9 ± 0,8 ab	10,5 ± 1,4 a	10,9 ± 1,2 b	12,8 ± 2,1 a	14,0 ± 2,7 b

Figure 4: *Height of IMI-resistant sunflower plants treated with the herbicide imazamox with or without supply of BCAA expressed in days after treatment (DAT). The values represent the mean of three biological replicates. Different letters (a, b, c) express significant differences (P < 0.05).*

3.4. Influence of BCAA addition in the root medium on physiological performance of imazamox-treated sunflower IMI-R plants

Experimental design

The experiments were carried out in climatic modules in a Fitotron chamber (H.V.A. – Koeling BVBA, Genk, Belgium) in the Hasselt University, Belgium. The seeds were rinsed in distilled water and placed for two hours in distilled water for imbibition. After that the seeds were placed in perlite for germination in 25 o C. When they reached the suitable size, they were betted on Styrofoam by sticky strip. The plants were placed in 2.5 liters pots (4 plants per pot) filled with ½ modified Hoagland nutrient solution.

The plants from hybrid Mildimi (IMI-R) were grown in a growth chamber with a 14/10 hours (light/dark) photoperiod, 250 μmol m−2 s−1 photosynthetic active radiation (PAR) at leaf level, temperature 24/22 ± 1 C° day/night and 60 % relative air humidity. The aeration was carried out by aquarium aeration system in each pot, night and day. At 4-6 thru leaf stage the plants were treated and were arranged an experimental design with four treatments:

1. Control – non-treated plants
2. BCAA – 0,5mM of each Branched chaun amino acids (Valine, Leucine and Isoleucine).
3. Imazamox – 40 g a.i. h-1 or approximately 132 μg per plant.
4. Imazamox + BCAA - 40 g a.i. h-1 or approximately 132 μg per plant + 0,5mM of each Branched chaun amino acids (Valine, Leucine and Isoleucine).

Each of the four variants was repeated in three pots (4 plants per pot).

3.3.1. Growth response and imazamox degradation

In general, the application of BCAA alone did not affect plant growth. On the 1st DAT there were no visible symptoms of herbicide phytotoxicity, which was also confirmed by biometric measurements, where no significant differences were observed (Fig. 5 A-C). However 7 DAT plants treated with imazamox alone demonstrated a sharp decrease in growth, whereas this was improved in plants treated with imazamox and BCAA together. The percentages of growth improvement are 21.9 %, 24.6 % and 30 % for fresh weight, dry weight and leaf area respectively. At the end of the experiment, 14 DAT, an inhibition of growth response of imazamox-treated sunflower plants was still notable, but in comparison with 7th DAT it was less pronounced. Adding of BCAA to imazamox-treated plants showed significant growth improvement also at 14 DAT, where the difference between single imazamox and combined treatment are 12.6 % and 17 % for fresh weight and leaf area respectively.

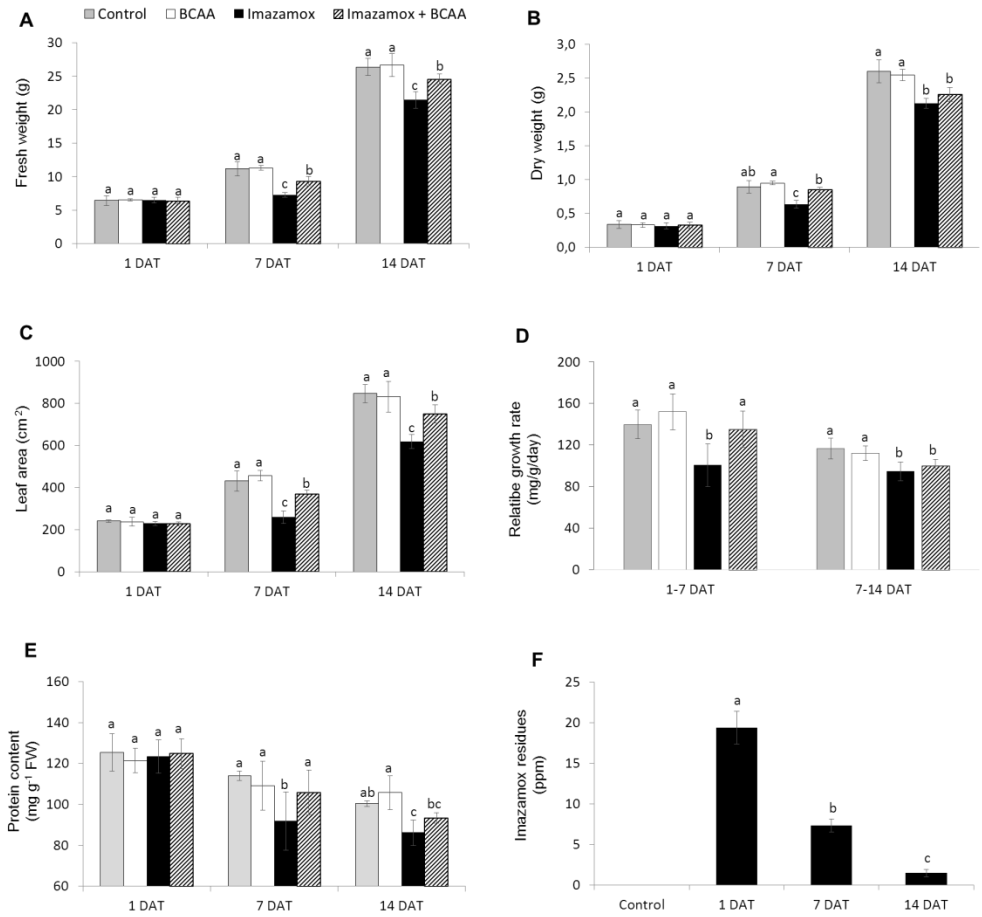


Figure 5: *Growth parameters (**A** - fresh weight, **B** - dry weight, **C** - leaf area), **D** - relative growth rate, **E** – Protein content and **F** – imazamox residues in sunflower plants treated with the herbicide imazamox alone. The values represent the mean of four biological replicates. In the graphs, different letters (a, b, c) express significant differences (P < 0.05). Error bars represent the standard deviation.*

For a more detailed analysis on the influence of imazamox and a combination of imazamox and BCAA on growth performance of CL sunflower hybrids we calculated their relative growth rate (Fig. 5 D). The results of the first tested period (1-7 DAT) showed a significantly decreased growth rate of imazamox-treated plants. At the same time, the growth rate of sunflowers that received a combined treatment with imazamox and BCAA was similar to this of control plants. For the period from 7 to 14 DAT the growth rate inhibition caused by imazamox application was still significant, but less pronounced. The growth rate of the plants treated with imazamox and BCAA was also retarded in this period.

No significant differences in total protein amount were detected between the different experimental conditions 1 DAT, while at a later stage (7 and 14 DAT) protein content was significantly decreased in imazamox-treated plants compared to the controls. In the plants treated with both imazamox and BCAA the decrease in protein content was less pronounced and not significantly different from control plants.

The imazamox residues in leaves of imazamox-treated sunflower plants 1, 7 and 14 DAT were measured (Fig. 5 F). The amount of imazamox in the plants significantly decreased over time from 19.38 ppm ± 2.03 (a) to 1.46 ppm ± 0.43 (c) 1 and 14 DAT respectively. Seven DAT the measured imazamox quantity was 7.32 ppm ± 0.79 (b).

3.3.2. Influence of BCAA on photosynthetic performance of imazamox-treated plants

To analyze the photosynthetic performance, leaf gas exchange parameters (Fig. 6), photosynthetic pigment concentrations (Table 3) and chlorophyll fluorescence parameters (Table 4) were determined. From leaf gas exchange measurements, it was obvious that photosynthetic rate (A) was inhibited by imazamox treatment and this inhibition was most notable (24.7%) 7 DAT compared to control values (Fig. 6 A). This inhibition was not caused by a decreased CO_2 concentration since there was no significant difference in CO_2 concentration at this time point (Fig. 6 C). Fourteen DAT there was still a significant inhibition of A, but it was less pronounced. The combined application of BCAA to the imazamox-treated plants resulted in a significant improvement of 15.6% of the photosynthetic rate compared to plants treated with imazamox alone 7 DAT. Interestingly the photosynthetic rate of plants with a single BCAA application was lower than the control values. No major alterations were observed in transpiration rate (Fig. 6 B) between the different experimental conditions, except that a single application of BCAA resulted in a slight decrease.

The concentrations of the photosynthetic pigments only showed a slight decrease in carotenoid (car) content of imazamox-treated plants 1 DAT (Table 3). Seven DAT the amount of pigments in the leaves of imazamox-treated sunflower was strongly decreased compared to the control with values of 31.5%, 19.1% and 22.3% for chl*a*, chl*b* and car, respectively. The pigment content in plants treated with a combination of imazamox and BCAA solution was also lower in comparison to the control plants, but this decrease was in general less pronounced than in the solely imazamox-treated plants (18.3%, 14.9% and 16.6% for chl*a*, chl*b* and car, respectively). A similar pattern was observed in plants 14 DAT. On the 14th DAT the chl*a* content was significantly lower in imazamox-treated plants compared to non-treated control plants (25.4% inhibition). The same was also with chl*b* and car where the inhibition rate was 24.8% and 18.1 respectively. Addition of BCAA to imazamox-treated plants resulted in a significant increase in chlorophylls compared to single imazamox treatment, with an improvement of 17.1% for chl*a* and 15.9 for chl*b*.

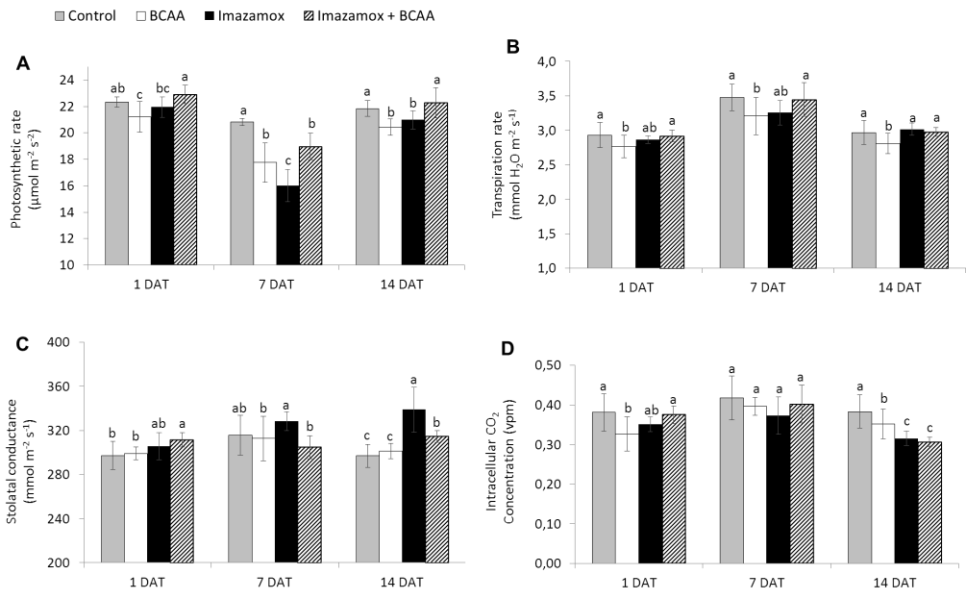


Figure 6: Leaf gas exchange parameters *(**A** – photosynthetic rate, B – transpiration rate, C – intracellular CO_2 concentration, D – stomatal conductance) of sunflower plants treated with the herbicide imazamox and branched chain amino acids added. The values represent the mean of three biological replicates. In the graphs, different letters (a, b, c) represent significant differences (P < 0.05). Error bars represent the standard deviation.*

Table 3: *Concentrations of photosynthetic pigments in sunflower plants treated with the herbicide imazamox and branched chain amino acids added. The values represent the mean of three biological replicates. In the table, different letters (a, b, c) express significant differences (P < 0.05).*

	Treatment	***1 DAT***	***7 DAT***	***14 DAT***
Ch a	*Control*	*1,36 ± 0,14 (a)*	*1,54 ± 0,23 (a)*	*1,91 ± 0,06 (a)*
	BCAA	*1,47 ± 0,16 (a)*	*1,27 ± 0,17 (b)*	*1,82 ± 0,11 (ab)*
	Imazamox	*1,30 ± 0,07 (a)*	*1,06 ± 0,08 (b)*	*1,43 ± 0,09 (c)*
	Imazamox+BCAA	*1,40 ± 0,14 (a)*	*1,26 ± 0,14 (b)*	*1,72 ± 0,10 (b)*
Ch b	*Control*	*0,41 ± 0,07 (a)*	*0,48 ± 0,08 (a)*	*0,60 ± 0,03 (a)*
	BCAA	*0,54 ± 0,07 (a)*	*0,43 ± 0,08 (ab)*	*0,55 ± 0,02 (ab)*
	Imazamox	*0,45 ± 0,04 (a)*	*0,39 ± 0,01 (c)*	*0,45 ± 0,04 (c)*
	Imazamox+BCAA	*0,47 ± 0,04 (a)*	*0,41 ± 0,02 (b)*	*0,53 ± 0,01 (b)*
Carotenoids	*Control*	*0,38 ± 0,04 (a)*	*0,40 ± 0,04 (a)*	*0,48 ± 0,02 (a)*
	BCAA	*0,42 ± 0,04 (a)*	*0,39 ± 0,03 (ab)*	*0,44 ± 0,02 (ab)*
	Imazamox	*0,36 ± 0,01 (a)*	*0,31 ± 0,01 (b)*	*0,39 ± 0,03 (b)*
	Imazamox+BCAA	*0,40 ± 0,03 (a)*	*0,33 ± 0,02 (b)*	*0,42 ± 9,94 (b)*

Table 4: *Parameters of chlorophyll fluorescence in sunflower plants treated with the herbicide imazamox and branched chain amino acids added. The values represent the mean of three biological replicates. In the table, different letters (a, b, c) express significant differences (P < 0.05).*

	Treatment	***1 DAT***	***7 DAT***	***14 DAT***
ETR	*Control*	*88,45 ± 1,3 (a)*	*101,55 ± 2,1 (a)*	*109,2 ± 0,6 (a)*
	BCAA	*83,4 ± 2,1 (a)*	*88,4 ± 2,0 (b)*	*99,45 ± 5,0 (ab)*
	Imazamox	*77,0 ± 4,3 (b)*	*56,0 ± 3,6 (d)*	*77,55 ± 10,1 (c)*
	Imazamox+BCAA	*85,05 ± 1,8 (a)*	*68,35 ± 1,4 (c)*	*90,8 ± 6,6 (b)*
Fv/Fm	*Control*	*0,838 ± 0,003 (a)*	*0,842 ± 0,001 (a)*	*0,832 ± 0,002 (a)*
	BCAA	*0,835 ± 0,001 (a)*	*0,841 ± 0,000 (a)*	*0,829 ± 0,014 (a)*
	Imazamox	*0,824 ± 0,007 (a)*	*0,787 ± 0,021 (b)*	*0,84 ± 0,003 (a)*
	Imazamox+BCAA	*0,834 ± 0,001 (a)*	*0,785 ± 0,016 (b)*	*0,84 ± 0,007 (a)*
qP	*Control*	*0,646 ± 0,02 (a)*	*0,676 ± 0,01 (b)*	*0,769 ± 0,02 (a)*
	BCAA	*0,639 ± 0,03 (a)*	*0,663 ± 0,00 (b)*	*0,713 ± 0,11 (a)*
	Imazamox	*0,641 ± 0,03 (a)*	*0,625 ± 0,02 (b)*	*0,710 ± 0,03 (a)*
	Imazamox+BCAA	*0,661 ± 0,02 (a)*	*0,813 ± 0,07 (a)*	*0,715 ± 0,03 (a)*
qN	*Control*	*0,634 ± 0,02 (a)*	*0,471 ± 0,00 (c)*	*0,446 ± 0,04 (bc)*
	BCAA	*0,570 ± 0,02 (b)*	*0,436 ± 0,04 (c)*	*0,634 ± 0,01 (a)*
	Imazamox	*0,617 ± 0,00 (a)*	*0,704 ± 0,07 (b)*	*0,498 ± 0,04 (b)*
	Imazamox+BCAA	*0,589 ± 0,01 (b)*	*0,786 ± 0,00 (a)*	*0,418 ± 0,02 (c)*

The electron transport rate of the sunflower plants was negatively influenced by the imazamox treatment throughout the entire experimental period, and this effect was mostly pronounced at 7 DAT. Addition of BCAA to imazamox-treated plants significantly diminished this negative effect on ETR, compared to the ETR values of single imazamox-treated plants (Table 4). The quantum yield of PSII (F_v/F_m) was significantly reduced at 7 DAT in plants treated with the herbicide. Photochemical and non-photochemical quenching (qP and qN) were also influenced by the herbicide, as the imazamox has higher effect on qN increasing it values from the 1 to 14 DAT.

3.3.3. Influence of BCAA on AHAS enzyme activity and *AHAS1* gene expression in imazamox-treated plants

The AHAS enzyme activity in IMI-R sunflower plants in our experimental setup was measured 1, 7 and 14 DAT. The results showed markedly decreases in AHAS enzyme activity after imazamox treatment from the 1st (80.6%) until the 14th DAT (61.1%), compared to the enzyme activity in control plants. The AHAS enzyme activity in plants treated with a

combination of imazamox and BCAA was similar and not statistically different to the enzyme activity in the plants treated with imazamox alone. Whereas a single application of BCAA did not influence significantly the AHAS activity at 1 and 7 DAT, while at 14 DAT it significantly diminished.

To establish whether expression of the *AHAS1* gene was directly affected by imazamox treatment and in combination with BCAA, we measured its relative gene expression. From the expression of *AHAS1* gene (Fig 6 B), it was obvious that plants treated with BCAA showed a down-regulation in the transcript level of the *AHAS1* gene at 1 DAT (BCAA, BCAA & imazamox application) and 7 DAT (BCAA application). The expression of *AHAS1* gene was not affected by both treatments 14 DAT.

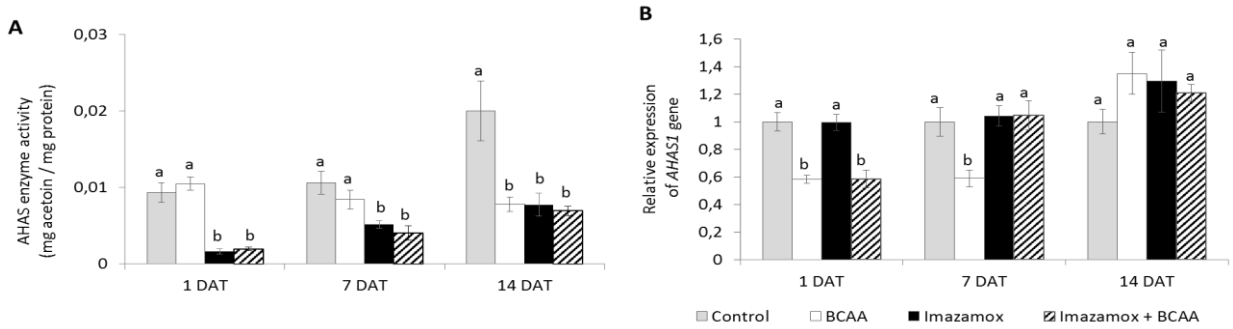


***Figure 7: A** - Acetohydroxyacid synthase enzyme activity and **B** - Relative expression of AHAS1 gene encoding the enzyme acetohydroxyacid synthase in sunflower plants treated with the herbicide imazamox and branched chain amino acids added. The values represent the mean of three biological replicates. In the graphs, different letters (a, b, c) express significant differences ($P < 0.05$). Error bars represent the standard deviation.*

3.5. Effect of leaf-applied biostimulant on imazamox-treated IMI-R sunflower plants

Experimental design

The experiments were carried out in climatic modules in a climatic modules in Agricultural University, Plovdiv. The seeds were rinsed in distilled water and placed for two hours in distilled water for imbibition. Than the seeds were placed in perlite for germination in 25 o C. When they reached the suitable size, they were betted on Styrofoam by sticky strip. The plants were placed in 2.5 liters pots (4 plants per pot) filled with ½ modified Hoagland nutrient solution.

The plants were grown in a growth chamber with a 14/10 hours (light/dark) photoperiod, 250 μmol m−2 s−1 photosynthetic active radiation (PAR) at leaf level, temperature 24/22 ± 1 C° day/night and 60 % relative air humidity. The aeration was carried out by aquarium aeration system in each pot, night and day. At 4-6 thru leaf stage the leaves of the plants were sprayed with imazamox and/or biostimulant and were arranged an experimental design with four treatments:

1. Control – non-treated plants.
2. Biostimulant (AAE), 1% Terra-Sorb® foliar - solution in volume approximately 1 ml per plant.
3. Imazamox – 40 g a.i. h-1 or approximately 132 μg per plant.
4. Imazamox + AAE - 40 g a.i. h-1 or approximately 132 μg per plant + 1 % Terra-Sorb® Foliar solution in volume approximately 1 ml per plant.

Each of the four variants was repeated in three pots (4 plants per pot).

Both, leaf chlorosis and deformations in young leaves developed in imazamox-treated sunflower plants. These symptoms were strongly pronounced at 7 DAT, when small necrotic spots appeared in the most injured leaves. At 14 DAT, the plants developed new leaves without visual symptoms of toxicity, but the latter subsisted in the older leaves of the sunflower plants.

Imazamox-treated plants were characterized by delayed growth. The growth inhibition was significant at 7 DAT with 42.5%, 29.6% and 48.4% decreased fresh weight, length and leaf area, respectively, in comparison to the untreated plants. At 14 DAT the growth inhibition in imazamox-treated sunflower plants was still significant, but less pronounced. Application of only amino acid extract (AAE) did not have any effects on sunflower plants at 7 and 14 DAT, but the growth of plants exposed to the combined treatment (AAE + imazamox) was less retarded and their performance was better as compared with that of imazamox-treated plants. This effect was limited at 7 DAT and more pronounced at 14 DAT.

3.5.1. Chlorophyll fluorescence

The data presented in Figure 8 (A) describing one-second induction transients of the relative variable fluorescence showed slight differences between the different treatments at 7 DAT. The transients show the typical steps of induction of ChlF: O – initial fluorescence level, J – is recorded when the rates of reduction and oxidation of QA become equal (2 ms after starting of illumination), I – recorded at 30 ms when the rate of reduction and oxidation of plastoquinone (PQ) are equal; P – maximal ChlF level, recorded at 300 ms when the PQ pool is fully reduced. Though the overall shape of the rise was highly similar for the different treatments, the steps of induction J and I were different.

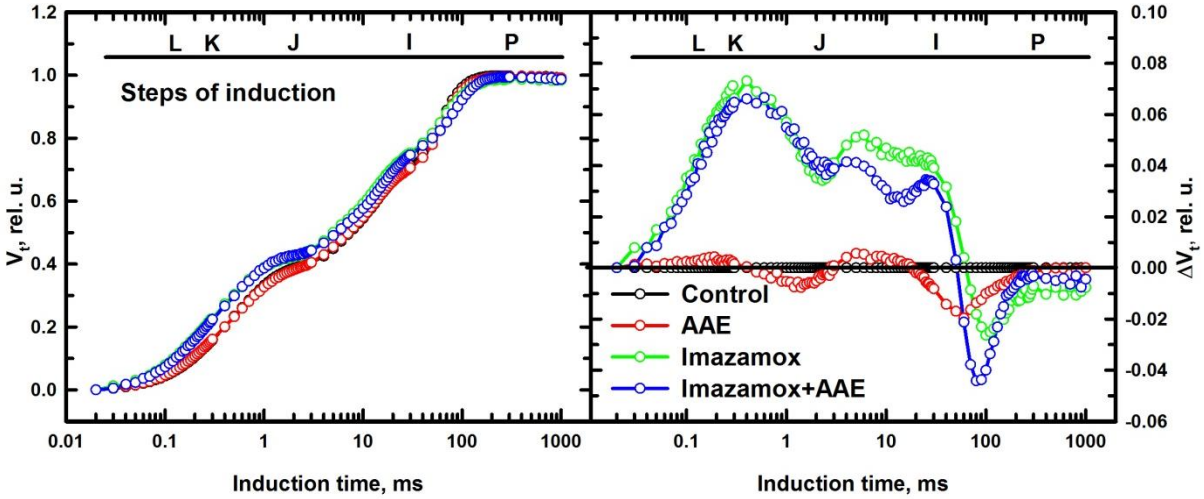


Figure 8: *(**A**) Relative variable fluorescence (Vt) transients recorded for 1 s with 3000 µmol m^{-2} s^{-1} PPFD after 1 h dark-adaptation of the measured spots on native leaves of imidazolinone resistant sunflower plants, exposed to single and combined treatment by imazamox and AAE, 7 DAT. Non-treated plants were used as controls. (**B**) Differential curves of relative variable fluorescence when the Vt values of ChlF rise recorded in control plants is subtracted from the corresponding values measured in treated plants.*

To visualize and analyze those differences throughout the induction time, the differential curves were calculated by subtracting the Vt curve of the untreated control from the curves recorded for the treated plants (Figure 8 B). The single imazamox and combined imazamox + AAE treatments showed positive ΔVt values from O until the induction transient I-P, where the ΔVt values turned negative until P (zero by definition). Moreover, the progress of both curves is very similar from O to J while different from J to I. Positive ΔVt values indicate lower rates, i.e. decreased efficiency of electron transport and negative values the opposite. The AAE treatment showed a fluorescence transient close to that of the untreated control. These findings indicate that imazamox had a prolific inhibition effect on the light phase photosynthetic reactions even if its specific site of action is not photosynthesis while

the biostimulant altered them just slightly. In addition, when added together with imazamox, a slight beneficial effect of AAE was indicated by the lower ΔVt values during J-I transient in comparison to the single herbicide treatment.

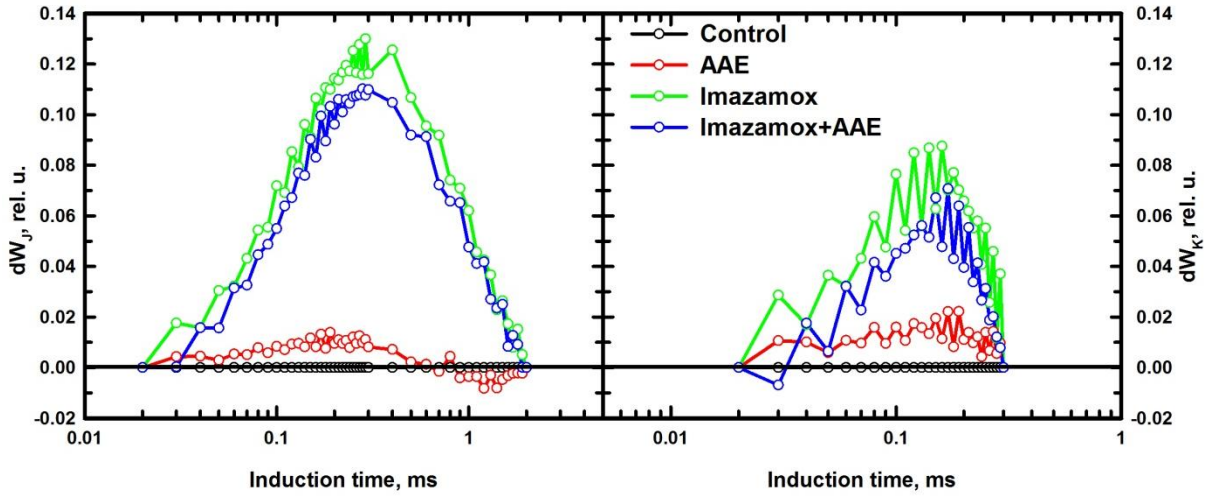


Figure 9: *Differential curves of relative variable fluorescence, double normalized from FO to FJ (**A**) and from FO to FK (**B**), acquired from native leaves of imizadolinone resistant sunflower plants, exposed to single and combined treatment by imazamox and AAE, 7 DAT. Non-treated plants were used as controls. Experimental conditions are the same as in Fig. 8.*

The differential curves composed from O to J (Figure 8 A) provide information about the balance of the electron transport through PSII. A pronounced positive peak at K was observed when imazamox was added alone or together with AAE. The positive K peak which is a sign for disturbances in the oxygen evolving complex is often observed during stress conditions (Strasser *et al.,* 2004). The differential curves constructed from O to K (Fig. 9 B) are associated with the level of energy transfer between antennae complexes of different RC, i.e. photosynthetic unit connectivity. Positive values at 0.1 ms are known as L band and indicate lower connectivity as was the case for the imazamox treatment.

OJIP test parameters were calculated from the ChlF transients (Figure 10). Once again the effect of the imazamox was obvious. Application of imazamox lead to higher FO, MO, ABS/RC, lower γRC and the almost unchanged RC/CS0 indicate more chlorophyll a pigments in the antenna that could not transfer their energy to a RC and thus emit fluorescence. This phenomenon can be attributed to the lowered φ(Po) and elevated DIO/RC, i.e. rise in the photochemically inactive PSII RCs. In addition, the increases of parameters t(FM), SM and N after imazamox treatment indicate increased relative numbers of electron acceptors in the PQ pool or at the PSI acceptor side per RC. We hypothesize that these observations are due to a

decreased de novo synthesis of reaction center proteins as a result of the inhibitory effect of imazamox on the branched amino acid synthesis.

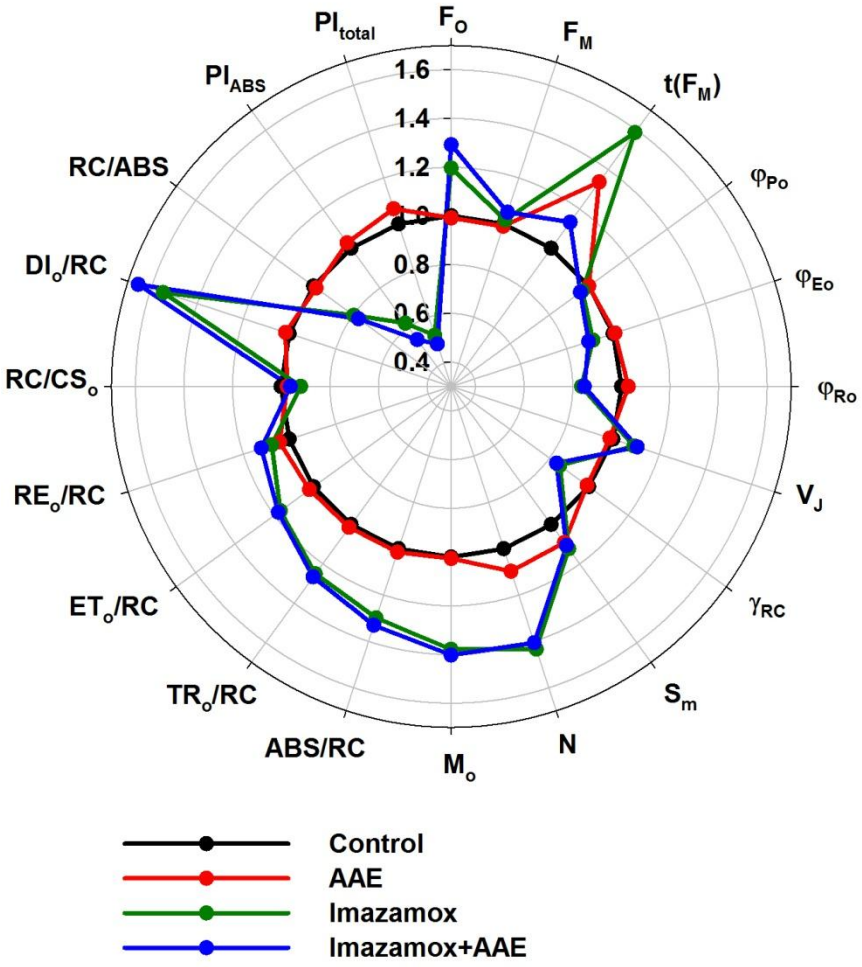


Figure 10: *OJIP test parameters derived from ChlF induction transients recorded from native leaves of imidazolinone resistant sunflower plants, exposed to single and combined treatment by imazamox and AAE, 7 DAT. Non-treated plants were used as controls. Experimental conditions are the same as in Fig. 8.*

φEo and φRo got lower in the imazamox-treated plants in correspondence to the higher ChlF values at J and I, resulting in decreases of both the PIABS and PItotal. These parameters summarize the fact that the efficiency of the photosynthetic light phase was negatively impacted by imazamox. As for the AAE action, the overall picture of the OJIP test parameters indicates that it did not alter the imazamox effect as well as the state of the photosynthetic machinery in the untreated control plants.

4. GENERAL DISCUSSION

Sunflower is one of the most important oilseed crops worldwide. Its cultivation occupies the fourth position in the world with approximately 25 million hectares sown annually and yields of 44 million tones (FAOSTAT). Weeds are among the most significant problems that farmers have to deal in sunflower cultivation, causing considerable yield losses. The proper weed management is a milestone for high yielding and quality sunflower production. Therefore, it is important to investigate the effect of the herbicides used in sunflower cultivation and their physiological effect on sunflower as a non-target plant species. With the conduct of the present studies we explore the physiological response of sunflower plants to the herbicide imazamox. It is a highly selective herbicide of the imidazolinine group which inhibits the branched chain amino acid biosynthesis by blocking the enzyme acetohydroxy acid synthase. A good solution for the weed problem in sunflower cultivation is the technology Clearfield, which is using the imidazolinine herbicides in combination with IMI tolerant sunflower hybrids.

4.2. Tolerance of IMI-R sunflower hybrids to the herbicide imazamox

The tolerant sunflower hybrids are the result of a naturally occurred mutation in wild type plants, after multiple treatments with AHAS inhibiting herbicides in soybean fields (Al-Khatib et al., 1998). As a result, after careful selection, a number of sunflower Clearfield hybrids have been developed and released to the agricultural marked. We tested the tolerance of a few IMI-R cultivars (LG 56.58, Tektonik, Alego, Mildimi and Primis) to the herbicide imazamox with the aim to select the most tolerant hybrid for further studies. The tolerance was evaluated by the modulation of the photosynthetic performance of plants treated with the recommended field dose and double dose of the herbicide imazamox with observations done on 7DAT and 14 DAT. The photosynthetic rate and photosynthetic electron transport rate were inhibited to a different extent in all hybrids on 7 DAT, while on 14DAT these values were similar to those of the control plants. We consider the measured differences in the photosynthetic performance between the tested hybrids on 7DAT as an indicator of different tolerance levels to the herbicide imazamox. The best photosynthetic performance was scored with the hybrid Mildimi which was used in our further studies.

4.3.Fast response of IMI-R and IMI-S sunflower hybrids to the herbicide imazamox

Plant tolerance to herbicides at the biochemical level depends on both specific and non-specific defence mechanisms. Usually, quick plant responses to different stress factors

are based on non-specific mechanisms. To identify to what extent both kinds of mechanisms are induced in sunflower plants by the herbicide imazamox we compared the responses of IMI-R and IMI-S sunflower hybrids 24 hours after the treatment.

Our results showed dissimilarities in the fast stress response and defense mechanisms in the compared hybrids. In the IMI-S sunflower hybrid Albena, the total amount of the metabolite glutathione was increased after imazamox application. These plants responded with activation of the antioxidative cell defense network manifested by enhanced antioxidant enzyme activities, such as GR, GROX and SPOD. In contrast, in IMI-R hybrids almost no changes in the antioxidative enzyme activities were observed, while GSTs activities were significantly higher. These results let us speculate that the *AHAS1* gene mutation, conferring the resistance to imidazolinone herbicides in IMI-R sunflower plants, leads to fast activation of the so called non-target mechanism of imazamox detoxification via glutathione-mediated system.

4.4. Monitoring the physiology alterations of imazamox-treated IMI-R sunflower plants

The Clearfield sunflower hybrids are resistant to the herbicides of the imidazolinones family and can survive doses which are lethal for the non-resistant weed plants. Despite the high selectivity of the herbicides and the high performance of the resistant sunflower hybrids a slight and transient growth inhibition can be observed after imazamox application, especially in combination with adverse environmental conditions during and after the herbicide spraying.

a) Growth response and imazamox degradation

To determine the periods of inhibition and recovery following imazamox treatment in IMI-R sunflower hybrids we measured the linear growth parameters like plant mass, height and leaf area. Our results demonstrate that the growth retardation was mostly pronounced on 7 DAT, while on 14 DAT the treated plants were already recovering their growth performance. The measurement of RGR showed that growth inhibition was mainly induced in the first tested period, while at the second period (7 – 14 DAT) it was still obvious, but much less expressed.

Nearly 90% of sunflower oil is used for human consumption and only 10% of the total production is utilized as a source for biodiesel and industrial applications, which is the main difference between sunflower and the other oilseed crops. Therefore, the detoxification rate

and amounts of remaining pesticides in the plant tissues and consequently in the produced oil is an issue of prime importance for public health.

In our study we investigated imazamox detoxification in the leaves of IMI-R sunflower plants. The obtained results showed that the metabolization of imazamox was very rapidly activated and effectively working since the amount of non-bound imazamox on the 14^{th} DAT was only approximately 10%. Rapid imazamox catabolism have been reported also in other IMI tolerant crops, such as wheat (Rojano-Delgado et al., 2014). A crucial player in the intensified imazamox metabolism in the tolerant plants is the metabolite glutathione, which is conjugating and detoxifying the imazamox molecules through reactions catalyzed by the GSTs enzyme family.

b) State of the antioxidative defense network

The imidazolinone herbicides have not been reported to cause considerable oxidative stress in target or non-target plants. The analyses of antioxidative enzymeactivities in our study demonstrated a slight increase in some enzymes such as SOD and SPOD, mainly on 7DAT. On 14 DAT the activities of those enzymes were similar to the ones in nontreated plants. These results support the hypothesis that this transitory activation of a few tested antioxidative enzymes was caused as a secondary effect following the inhibition of protein synthesis caused by imazamox application. In addition, an increased amount of total glutathione was observed after imazamox application on 7 and 14 DAT, but the GSH/GSSG ratio of the imazamox-treated sunflower plants was in the range between 96 – 99 %, which is considered as a normal level and does not support induction of the antioxidative defense mechanisms. This was also confirmed by the activity of the GR enzyme, which was also not increased as a result of the imazamox application. All these results indicate that slight oxidative stress could be observed as a secondary effect of imazamox treatment and impaired protein synthesis.

c) Photosynthetic performance

Photosynthesis is related with many others processes in the cell and is very sensitive to changes in the conditions, including pesticide treatment or stress factors. Therefore photosynthetic parameters, such as gas exchange or chlorophyll fluorescence, of the plants are good indicators for the overall plant performance. In the current work we analyzed the photosynthetic performance of imazamox-treated IMI-R sunflower plants.

Our data clearly demonstrated that in our experimental conditions the herbicide imazamox caused a transient inhibition of the photosynthetic performance of sunflower IMI-R

plants. The negative impact was obvious in both light-dependent photosynthetic redox reactions and leaf gas exchange processes. The inhibitory effect of imazamox on net photosynthetic rate of sunflower plants was the most pronounced on 7 DAT. This was partly due to decreased stomatal conductance for CO_2 uptake, lowered chlorophyll content as well as less intensive photosynthetic electron transport processes in thylakoid membranes. Using a sensitive method, based on precise time-resolution measurements of the fast photoinduced changes of chl *a* fluorescence, emitted mainly by the antennae pigments of PSII (OJIP test parameters) (Papageorgiou and Govindjee 2004), we detected some specific aspects of the imazamox-photosynthesis interactions related to both structure and function of the photosynthetic machinery. We found that the concentration of active PSII reaction centers in imazamox-treated plants was slightly diminished and the relative part of active reaction centers compared to the total chlorophyll content significantly decreased. The appearance of chlorosis in the leaves of imazamox-treated sunflower plants in our study was due to their significantly lower chlorophyll content as compared to the control. Although inhibition of photosynthesis was not a mode of action of imazamox, the results of our study showed suppression of photosynthetic gas exchange in sunflower plants caused by imazamox treatment. Kinetic measurements of the photosynthetic performance of imazamox-treated sunflower plants over time (1, 7 and 14 DAT) revealed a tendency to recovery.

4.5. Influence of BCAA addition in the root medium on physiological performance of imazamox-treated sunflower plants

The imidazolinone herbicides act through inhibiting the activity of the key enzyme AHAS involved in the branched chain amino acid biosynthetic pathway. Addition of BCAA to plants or cell cultures treated with AHAS inhibiting herbicides contributes to the alleviation of the growth retardation symptoms induced by these herbicides (Ray 1984). In our study we found that the supplementation of the growth medium of imazamox-treated IMI-R sunflower plants with BCAA reduced the negative effect of imazamox on their linear growth. The positive effect of BCAA was also supported by the data from the leaf area, fresh and dry weight measurements. The relative growth rate also confirmed the improvement of plant mass accumulation in plants receiving combined treatment of imazamox and BCAA, compared to the plants treated only with imazamox. In addition, BCAA applied to the roots influenced positively the photosynthetic performance on the imazamox-treated plants, based on the results from gas exchange and chlorophyll fluorescence analyzes. The pigment profiling also showed ameliorative effect of the additional application of BCAA to imazamox-treated

plants, in which the specific chlorosis caused by the herbicide was much less pronounced. Considering the fact that the total protein content in the sunflower plants subjected to single imazamox treatment was significantly lower compared to the plants receiving imazamox in combination with BCAA, we conclude that this positive effect could be due to the facilitation of *de novo* protein synthesis, which is hampered by the deficit of these tree BCAA in imazamox-treated plants.

4.6. Effect of leaf-applied biostimulant on imazamox-treated IMI-tolerant sunflower plants

The performance of plants exposed to different stress factors, including herbicides, could be improved by the use of a new group of agricultural products called biostimulants (Calvo et al., 2014). Therefore, we explored whether the application of PHs-based biostimulants could ameliorate plant growth and photosynthetic performance of imazamox-treated IMI-R sunflower plants. Considering the obtained results we can confirm that combined application of imazamox and the amino acid extract diminished the negative effects of the herbicide. For example, the OJIP test indicated lower rates, *i.e.* decreased efficiency of electron transport in the plants treated with imazamox. Moreover, the AAE treatment showed a fluorescence transient close to that of the untreated control. These findings indicate that imazamox had a pronounced inhibitory effect on the light phase photosynthetic reactions even if its specific site of action is not photosynthesis itself while the biostimulant altered them just slightly. In addition, when added together with imazamox, a slight beneficial effect of AAE was indicated by the lower ΔVt values during J-I transient in comparison to the single herbicide treatment.

5. CONCLUSIONS

1. Treatment of Clearfield sunflower hybrids with the recommended field dose imazamox (48 g a.i. ha^{-1}) causes a transient and recoverable inhibition of plant growth and photosynthetic performance, while the doubled dose leads to significant damages and incomplete recovery.

2. The IMI-R sunflower hybrids LG 56.58, Tektonic, Alego, Mildimi and Primis differ in their tolerance to the herbicide imazamox. Assessment of the physiological status of imazamox-treated plants assigns the highest tolerance to the hybrid Mildimi.

3. IMI-R and IMI-S sunflower hybrids differ in the mechanisms involved in the rapid responses to imazamox. The IMI-S hybrid Albena is activating the cell antioxidative defense system, while in the IMI-R hybrid Mildimi detoxification mechanisms, including enzymes such as GSTs are triggered.

4. The IMI-R hybrid Mildimi detoxifies imazamox efficiently. The imazamox content in the leaves decreased with up to 90% in a period of 14 days.

5. Application of imazamox inhibits the leaf gas exchange in sunflower plants. The effect is detectable from the first DAT and most pronounced at 7 DAT; after that, the inhibition of the photosynthetic activity gradually decreases and two weeks after the treatment it reaches a level close to untreated plants.

6. The herbicide imazamox diminished significantly the activity of AHAS enzyme, but does not affect its regulation at the gene expression level. The addition of BCAA to the root medium decreased both AHAS enzyme activity and *AHAS1* gene expression, which might be explained as a result of a negative feedback downregulation.

7. The addition of BCAA to the root medium of imazamox-treated sunflower plants improved their growth and photosynthetic performance, which could be due to a facilitation of protein turnover.

8. The herbicide imazamox clearly affects the light dependent photosynthetic redox reactions. It diminishes the content of photosynthetic pigments, the concentration of active PSII reaction centers and disturbs the interactions between antennae complexes of PSII.

9. Application of the amino acid extract to imazamox treated plants diminished the negative effects of the herbicide. This is illustrated by both, a better photosynthetic performance and growth of the treated plants.

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Scientific achievements

1/ An overall picture of imazamox-induced injuries to sunflower, their consequences and recovery of the plants have been detailed by time-course monitoring on the physiological responses of the plants subjected to both separate and combined application of imazamox and branched chain amino acids.

2/ A novel fact, established in the conducted study, is the obtained evidence that the metabolite glutathione participates in the herbicide imazamox detoxification in sunflower plants by conjugation reactions catalyzed by the glutathione S-transferase enzymes family. This fact complements the known detoxification pathway of imazamox in plants by cytochrome P_{450} monooxygenase and glycosyl transferases.

3/ Using a sensitive chlorophyll *a* fluorescence method (OJIP) several novel aspects of imazamox-photosynthesis interactions have been described, in particular that imazamox slightly diminished the concentration of active PSII reaction centers and significantly decreased the relative part of active reaction centers compared to the total chlorophyll content.

Recommendation for the practice

Based on the obtained results demonstrating a positive effect of an amino acid extract (commercial product Terra-sorb) on imazamox-treated sunflower plants, we recommend it for foliar application in a rate of 3 L / ha given at 2-3 leaf pair for improving their growth and photosynthetic performance.

List of publications:

1. Balabanova D. and Vassilev A (2015) Response of sunflower Clearfield hybrids to both recommendable and higher doses of imazamox herbicide. Agricultural Sciences, 8, 18:41-46.

2. Balabanova DA, Paunov M, Goltsev V, Cuypers A, Vangronsveld J and A Vassilev, (2016) Photosynthetic Performance of the Imidazolinone Resistant Sunflower Exposed to Single and Combined Treatment by the Herbicide Imazamox and an Amino Acid Extract. Front Plant Sci. DOI: 10.3389/fpls.2016.01559.

3. Balabanova D, Remans T, Vassilev A, Cuypers A and J Vangronsveld, Response of sunflower glutathione-mediated detoxification system to the herbicide imazamox. Submitted to Acta Physiologiae Plantarum.

Participation in scientific conferences:

Balabanova, D., Remans T., Vassilev A., J. Vangronsveld and A. Cuypers (2014). Response of sunflower glutathione-mediated detoxification system to the herbicide imazamox. 19th Plant Biology Europe FESPB/EPSO Congress. Dublin, Ireland. June 22nd to 26th 2014. Abstract of poster presentation.

Balabanova D. and A. Vassilev (2015) Response of sunflower Clearfield hybrids to both recommendable and higher doses of imazamox herbicide. Jubilee scientific conference 70th Anniversary Agricultural University – Plovdiv. Abstract of oral presentation.