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Oxidative stress level and dehydrin gene expression pattern differentiate two contrasting cucumber F1 hybrids under high fertigation treatment

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Abstract

Two cucumber F1 cultivar hybrids were investigated for stress tolerance markers upon application of different strength of Hoagland fertigation solutions (HG). ‘Joker’ and ‘Oitol’ cultivar hybrids were studied, representing typically field grown and greenhouse cultivated genotypes, respectively. At standard fertigation level (0.5 × HG) in controlled environment young ‘Joker’ plants displayed slower growth than ‘Oitol’ based on total leaf area. At this basal nutrient concentration leaves of 'Joker' plants had significantly lower antioxidant capacity and

higher malondialdehyde (MDA, an indicator of lipid peroxidation) level than ‘Oitol’. According to RT-qPCR transcript levels of several antioxidant enzymes’ genes (ascorbate peroxidase, glutathione reductase and glutathione peroxidase) were significantly higher in ‘Joker’ compared to ‘Oitol’. At increased HG concentrations (1.0, 1.5, 2.0, and 2.5 × HG) growth didn’t change significantly in either hybrid. Osmotic potential declined at higher fertigation levels. Antioxidant capacity increased in both hybrids with strong characteristic differences favouring ‘Oitol’ plants. Higher MDA content of leaves testified more oxidative burden in 'Joker' plants at all and especially at the more concentrated HG treatments. This trend was also approved by results of bio photon emission imaging, which is a powerful method to quantify stress level in living tissues with autoluminescence detection technology. Gene expression for antioxidant enzymes followed HG concentration-dependent increase in both hybrids, at a substantially higher level in 'Joker'. Expression of the dehydrin gene *DHN3* was preferentially induced at elevated fertigation levels in ‘Oitol’ plants, which could contribute to the lower oxidative stress detected in this hybrid. Results presented in this report demonstrate differences in shoot growth, antioxidant capacity, level of oxidative stress and antioxidant gene expression in two contrasting cucumber hybrids at basal fertigation. Furthermore, excessive HG fertigation was found to increase oxidative stress in a genotype-specific way. This effect may be due to different antioxidant capacity and differential expression of stress protective genes, such as the *DHN3* dehydrin.

Keywords: Cucumber, Fertigation, Antioxidant genes, Nutrition stress, Dehydrin, Biophoton emission

Introduction

Fertigation is indispensable for efficient and productive agricultural systems. Many reports emphasize the environmental consequences of excessive fertigation such as secondary soil

salinization and contamination of water resources. Nevertheless, one cannot deny the necessity of a balanced mineral diet for crop plants, which is routinely delivered by excessive fertigation. Adequate fertigation solutions have been developed and recommended for common crops mostly considering maximized yield and to a lower extent the taste and quality of the products [1]. A correlation between elevated concentrations of components of fertigation solutions and an increase of stress level is becoming evident in some plant species [2]. However, little is known about molecular details and intra species variations of stress triggering potential of complete fertigation solutions, although it is of paramount importance also from a practical point of view.

Plants sense the environment and adapt their metabolism accordingly by utilizing their biological resources [3]. Stress tolerance mechanisms in plants are believed to involve regulated ion transport processes, which may modulate signalling pathways of the adaptive mechanisms to implement transcriptional and translational control of relevant genes [4]. Different indicators have been identified reflecting the stress level of plants by phytochemical analysis of the molecules generated during lipid peroxidation and oxidative metabolism. Another powerful method to observe and quantify the stress level in living tissues is the autoluminescence detection technology. It can monitor the electronically excited molecular species of cellular processes; giving a broader perspective to determine the stress level and dynamics in plants thus allowing for the opportunity to unravel a broad range of stressors be either abiotic or biotic (including stressors posing stress in ”latent” form e.g. by hidden lifestyle arthropods [5,6]. The power of this method lies in its non-invasive and label-free nature hence allowing for monitoring oxidative processes in crop plants at real-time. Up regulation of some marker proteins such as dehydrins (DHN) and the expression of their corresponding genes were also considered to reveal the status of osmotic stress [7]. Dehydrins (DHNs) belong to a distinct class (II) of the late

embryogenesis abundant (LEA) proteins [8]. DHNs are abundant during late embryogenesis and also are inducible in vegetative tissues upon several stresses, including dehydration [9]. Several reports have identified correlation between stress tolerance and higher expression of DHNs [10-13]. The trends were further supported when overexpression of DHNs resulted in more stress tolerant crops [14-19]. In spite of this positive correlation, there have been also published contrasting cases in which overexpression of some DHNs did not enhance stress tolerance in general [20,21] DHN proteins however, are able to bind membranes [7] and protecting them from lipid peroxidation [22]. Therefore, the contribution of DHNs to cope with different components of abiotic stressors such as oxidative stress needs to be addressed with specific experiments designed for each species and even cultivars.

Cucumber is one of the most important vegetables with constantly growing vegetation size. The global production of cucumbers in 2017 was more than 83 million tons produced on areas of approximately 2.2 million hectares [23]. Excessive fertigation, which is often a practical issue in cucumber production units, has not been addressed from the perspectives of molecular biology. The experiments presented here were designed to investigate the potentially stress-inducing effect of elevated fertigation on cucumber by comparing some physiological parameters of two commercial F1 hybrid cultivars under such treatments. As concentrated HG solutions exert osmotic stress, but do not deliver sodium or chloride, this stress component can be implied as the main stressor arising from these treatments. Several parameters were measured or detected to study especially the oxidative component of the stress responses triggered in the two hybrids, also aimed at elucidating potential intra species variability of the responses. Expression of some stress associated *DHN* genes was also quantified and the results were discussed.

Materials and methods

Plant materials and experimental conditions

Two cucumber F1 cultivar hybrids; 'Joker' (open field grown for pickling) and 'Oitol' (greenhouse grown slicing type) were studied in this research. F1 seeds of 'Joker' and 'Oitol' were obtained from ZKI Ltd., Hungary and Semillas Fito Co., Spain, respectively. To establish the culture, cucumber seeds were washed in running tap water, then surface sterilized by being immersed in 0.5% (v/v) sodium hypochlorite solution for 10 min followed by rinsing thoroughly with distilled water for 3 times and finally submerged in 100 ml of distilled water for 24 hours at 25°C to imbibe. For each treatment four seeds were planted in 7.5 × 7.5 × 6.5 cm rockwool cubes (in triplicate) and inserted in 20-cm-diameter pots containing 120 g of perlite and kept in dark for two days at 25°C. Pots were transferred into a light room where seeds germinated and grew at 26 ± 1 ºC under a 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 150 $\mu mol.m^{-2}.s^{-1}$ at culture level (provided by cool-white fluorescent lamps) and at 50-55 % of relative humidity. Fertigation treatments were applied every other day after cotyledon's expansion and emergence of the first leaf. The treatments were continued for 21 days when leaves of each individual plants of all treatments were harvested, photographed and total leaf area per plant was calculated with ImageJ software (ImageJ, Image Processing and Analysis in Java, USA, downloaded: 2016.10.12.) and the obtained data were statistically analysed. Two individual plants in each pot were kept intact and analysed with biophoton emission imaging. Fully expanded leaves were selected from each treatment, placed in sterile vials, labelled and deep frozen in liquid nitrogen for further molecular analysis. All subsequent experiments were performed at least twice on different biological material with similar results.

Fertigation treatments

Different concentrations of Hoagland solution [24] were prepared according to a modified formulation [25] due to the higher nitrogen demand of cucumber plants [26]. The phosphorus content was also modified to a level generally applied in plant cultures [27]. Treatment of plants started after expansion of cotyledons when pots were supplied with 250 ml of Hoagland solutions of different strengths (0.5, 1.0, 1.5, 2.0, and 2.5 ×) containing 0.5 mM MES buffer as shown in Table S1 of supplementary material. Pots were flashed with fresh fertigation solution every other day, which created a semi-hydroponic environment for the plants. The pH of the treatment solutions were measured daily and adjusted to 5.8-6 by adding 1 M H_2SO_4.

Determination of osmotic pressure (OP)

The osmolarity (c) of the cucumber leaves cell sap was determined according to [28] using a freezing-point micro-osmometer (Osmomat 030-D; Gonotec, Berlin, Germany). The fresh leaves of cucumber plants (0.5g) grown under different strength of Hoagland fertigation in triplicate experiments were collected and frozen in liquid nitrogen. The frozen samples (from three different plants of each treatment) were grounded in pre cooled mortar with pistils of which 0.1g were added to 500 µL of Milli-Q water, then vigorously vortexed for 30 seconds and centrifuged for 30 minutes at 14000 rpm. The supernatants were collected and used for osmolytes content determination with 5 technical replicates for each biological sample. OP was calculated from the mosmol kg^{-1} using the formula: OP (MPa) = $-c$ (mosmol $kg)^{-1} \times 2.58 \times 10^{-3}$ [29].

Extent of lipid peroxidation

Malondialdehyde (MDA) content was determined by thiobarbituric acid (TBA) reaction following the original method of Heath and Packer [30] with some modifications. Samples of 0.6 g (0.1 g fresh plant material from six individual leaves of each treatment) were homogenized with 2 ml of 0.1% trichloroacetic acid (TCA) in cold mortars from which 1.8 ml was transferred

to Eppendorf tubes. To this solution, 40 µl of 20% butylated hydroxytoluene (BHT) in absolute ethanol was added to stop further lipid peroxidation [31]. The solutions were vortexed for 15 s and centrifuged at 13000 rpm for 10 min at 4 ºC. From the clear supernatant 0.25 ml was added to 1 ml of 20% TCA containing 0.5% TBA, gently mixed and briefly centrifuged for 5 s. The solutions were incubated in a block heater for 30 min at 96 ºC. The reactions were stopped by cooling the solutions immediately on ice, followed by centrifugation at 10000 rpm for 5 min. Absorbance at 532 and 600 nm was recorded using a microplate spectrophotometer (PowerWave XS2, BioTek, USA) and MDA concentration was calculated by subtracting the non-specific absorption at 600 nm from the absorption at 532 nm by using the absorbance coefficient of extinction 156 mM^{-1} cm^{-1}. Finally, the results were expressed as nmol g^{-1} fresh weight (FW).

Determination of antioxidant capacity

The level of antioxidant capacity in the leaf tissues of cucumber plants was evaluated using the FRAP assay following the method of Benzie and Strain [32]. Frozen leaf samples (0.2 g) were grinded in 70% ethanol (2 ml) for extraction and subsequent centrifugation (10 min at 14000 rpm). Aliquots of 50 µl from the supernatants were mixed with 250 µl of freshly made FRAP reagent solution (10 mM TPTZ solution in 40 mM HCl; 20 mM $FeCl_3$ and 0.3 M acetate buffer, pH 3.6 in 1:1:10 ratio) in flat bottom 96-well microplates. Absorbance was measured at 593 nm at 0 and after 6 minutes of incubation at 37 ºC with a microplate spectrophotometer. Ascorbic acid standards (100-1000µM; R^2: 0.9988) were processed in the same way to generate a calibration curve. Results were calculated with the following formula and expressed as means of three replicates from each set of biological samples ± standard deviation:

FRAP value of Sample (µM AA eq. g^{-1}FW) =

(Change in absorbance of sample from 0 to 6 minutes / Change in absorbance of Ascorbic acid from 0 to 6 minutes) × FRAP value of Ascorbic acid (1000 µM).

Ultra-weak bio photon emission

Leaves of cucumbers grown under different fertigation treatments were subjected to UPE (ultra-weak photon emission)-imaging. Intact leaves of approximately same size were separated from the plants and placed in the NightShade LB 985 Plant Imaging System (Berthold Technologies, Bad Wildbad, Germany). Luminescence emissions in leaves deriving from the test plants were imaged using a highly sensitive, thermoelectrically-cooled (-70 ºC) CCD camera (NightOWLcam, Berthold Technologies) mounted on a dark, light-tight camber. A back-lit, midband-coated full frame chip with a spectral range of 350 – 1050 nm (quantum efficiency: 90 % at 620 nm) was employed for photon detection and XY-imaging. In order to increase detection sensitivity the variable binning was set to: 2 x 2 resulting in final resolution of 512 x 512 pixels and 26 x 26 µm² pixel size (slow scan mode). The exposure time was: 60 sec and for image analysis the IndiGo software (Software Version 2.0.5.0, Berthold Technologies, Germany) was used. The presented images are selected from series of taken photos and represent the highest detected signal intensity level in case of each treatment.

RNA isolation, cDNA production, RT-PCR and RT-qPCR

Deep frozen leaf samples (0.5 g each) were ground in liquid N_2 using sterile mortar and pestles for total RNA extraction according to a CTAB-based protocol [33]. The RNA quality was determined on an EcoSafe-stained 1% agarose gel. RNA concentration was assessed using NanoDrop 1000 spectrophotometer at 260 nm. To eliminate genomic DNAs, all samples were treated with DNase I (Thermo Scientific) then RNA concentrations were normalized to 5 µg/30 µl for all reaction mix. DNase treated RNAs integrity was ensured on 1% agarose gel before

reverse transcription. First-strand cDNAs were synthesized by RT–PCR using 5 µg of total RNA as template and M-MuLV RT enzyme using Maxima Reverse Transcriptase kit (Thermo Scientific) with oligo $(dT)_{20}$ primers according to the manufacturer's protocol. Primers of cucumber ascorbate peroxidase, glutathione reductase, glutathione peroxidase and four different cucumber specific dehydrin along with a control actin gene (Table 2) were tested for PCR amplification with Go Taq G2 DNA polymerase (Promega, USA). Amplification was achieved by applying 30 reaction cycles in a Master Cycler instrument (Eppendorf AG, Hamburg, Germany) of 3 min at 95°C and 30 cycles of 30 s: 95°C, 60 s: 58°C, 30 s: 72°C and a final extension for 7 min at 72°C. Amplified fragments from genomic and cDNAs were visualized on 1.4% (w/v) ethidium bromide-stained agarose gel in 1×TBE. RT-PCR products of the putative dehydrin and actin genes were purified from agarose gels by using Viogene Gel Advanced Kit (Viogene BioTek Corp., Taiwan) cleaned with ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, USA) and sequenced in ABI PRISM 3500 Genetic Analyzer (Applied Biosystems) using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and gene specific primers.

RT-qPCR was performed in a CFX 96 Real-Time PCR System (Bio-Rad, USA) using SsoAdvanced Universal Inhibitor-Tolerant SYBR® Green Supermix (Bio-Rad) for fluorescence detection in 96-well optical plate format. The total volume of each qPCR reaction was 10 µL, containing 1 µL of cDNA, 4 µL of super mix, 0.5 µL (100 uM) of forward and reverse primers and, 4 µL of PCR grade water. PCR amplification was initiated with polymerase activation and DNA denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 30 s. A melting ramp was performed (65-95°C) at the end of the program to assess the PCR product specificity. To determine the PCR efficiency, the Cq

values of the standard dilutions from cDNAs were extracted for each primer pair and plotted to create a standard curve using Bio-Rad CFX Maestro software (Fig. S1 of supplementary material). A cucumber *Actin-3* gene (DQ115883) was used as endogenous control after its approved stability by Bio-Rad CFX Maestro software. The stability of *Actin 3* was also tested where assumptions for normality regarding the residuals were checked by Shapiro–Wilk' test and homogeneity of variances was tested by Bartlett's test using software R-Studio (Version 3.5.1.) as presented in Fig. S2 of supplementary material. Fold changes of the examined genes expression were estimated by the $2^{-\Delta\Delta Ct}$ method [34].

Statistical analysis

Obtained results were analysed using the R (R Development Core Team) software [35] and were shown as mean values with standard deviations among three biological replicates. The assumptions implied in statistical analysis were that the residuals should be normally distributed and that there is homogeneity of variance if *P* value is higher than 0.05 ($p>\alpha$) ($\alpha=0.05$). Normality of the residuals was proved by Shapiro-Wilk's test ($p>0.05$). Homogeneity of variances was checked by Levene's test. The differences among the means were evaluated by one-way ANOVA and considered significant at $P<0.05$. For the data analysis and representations, MS Excel 2013 was used.

Results

Plant growth and morphology

Growth of two cucumber F1 cultivar hybrids was monitored during Hoagland fertigation treatments. All plants grew normally with no visible sign of severe nutrient deficiency or stress. Different levels of fertigation did not result in any drastic change of the plants appearance in any of the studied cultivars throughout the experimental period. Total leaf areas of plants from each

treatment were recorded and the results are presented in Fig. 1. In general 'Joker' plants developed significantly smaller canopy under all treatment levels when compared to 'Oitol' at day 21 after germination. This was largely independent of the level of fertigation at the range of the nutrients supplied. Although the leaves area in the studied plants did not change significantly in response to even high fertigation concentrations, it decreased nominally at the 2.5 × HG treatment in 'Oitol' (Fig. 1).

Lipid peroxidation, osmotic potential, and antioxidant capacity in leaves

The leaf samples of both cucumber hybrid cultivars under elevated fertigation treatments were subjected for Malondialdehyde (MDA) which is often used as a marker for oxidative stress in plants. As presented in Fig. 2a., significantly higher MDA concentrations (p<0.05) were recorded in 'Joker' cultivar in comparison with 'Oitol' at all applied fertigation levels. At the same time there were significant differences of MDA content among fertigation treatments within both cultivars. A trend for positive correlation between peroxidation level and concentration of nutrients supplied could be established in both 'Joker' and 'Oitol'. Differences among MDA content were significant between 0.5 × HG and 2.0 × HG, with non-significant increments at concentrations in between (Fig. 2a). The increase in fertigation level from 2.0 × HG to 2.5 × HG resulted in a significant rise of MDA content (p<0.05) in both hybrids.

In order to clarify the osmotic relations in plants under the applied treatments, leaf osmotic potential was determined. Significant differences were found between the two cultivars and also among different Hoagland fertigation treatments (Fig. 2b). In both hybrids the lowest and highest osmotic pressures were recorded from plants treated with the lowest (0.5 × HG) and the highest (2.5 × HG) concentration of Hoagland solution; resulting in the highest and the lowest osmotic potentials, respectively. Therefore, in general an inverse relationship has been observed between

increased Hoagland concentration and decreasing leaf osmotic potential. There were however no significant differences (p<0.05) in osmotic potential of 'Oitol' leaves under 1.0, 1.5, and 2 × Hoagland treatments.

To search for additional factors playing role in the redox status of the leaves in the hybrids, determination of antioxidant capacity was conducted by FRAP assay. As presented in Fig. 2c, significant differences were found in antioxidant capacity between the two hybrids and among different fertigation treatments (p<0.05). Antioxidant capacity was significantly higher in 'Oitol' plants as compared to 'Joker' under each corresponding fertigation levels. The antioxidant capacity was not significantly changed when the Hoagland solution concentration increased from 0.5 × HG to 1.0 × HG in 'Oitol' and 'Joker' but application of higher nutrient levels (1.5, 2.0 and 2.5 × HG) resulted in significant changes (p<0.05) in both cultivars (Fig. 2c).

Ultra-weak bio photon emission

Ultra-weak bio photon emission analysis revealed high resolution significant differences between the two studied cultivars that were not observable by naked eye. Differential oxidative damage could be visualized by the imaging, which again gave stronger signals in 'Joker', indicating high oxidative stress particularly at more concentrated nutrient exposure (Fig. 3 and 4). The calculated photon Count per Second (CPS) values showed no significant differences (p<0.05) for Oitol plants treated with elevated Hoagland solutions indicating a higher capacity to cope with concentrated nutrients. Joker plants on the other hand showed significantly higher CPS values between the 0.5 × HG and 1.0, 1.5, and 2.0 × HG. The highest CPS values were recorded for Joker plants treated with 2.5 × HG which was significantly higher than that in all other treatments (p<0.05).

RT-PCR and RT-qPCR of cucumber antioxidant and dehydrin genes

Fragments of selected cucumber glutathione reductase (*GRI*), glutathione peroxidase (*GPXI*) and ascorbate peroxidase (*APXI*) were PCR amplified from reverse-transcribed complementary DNAs. Obvious higher expressions of *CsGRI* and *CsGPXI* have been found in the samples of 'Joker' plants (Fig. 5). As could be seen on the agarose electrophoresis gel, the expressions of both genes are upregulated as results of increased fertigation level. In the case of *CsAPXI*, the highest expression was observed when the plants were treated with 2.0 × HG solution. A clear difference was observed between the 'Joker' and 'Oitol' plants treated with the basic level of fertigation (0.5× HG).

In a search of potential clues to explain the observed physiological differences between the two cultivars, the transcription of dehydrin (*DHN*) genes was targeted. Cucumber *DHN* genes have been recently identified and reported with inducible expression under abiotic stresses [36]. Four different *DHN* genes (*CsDHN1, CsDHN2, CsDHN3*, and *CsDHN4*) of *C. sativus* were studied for their expression pattern from cDNA pools generated by reverse transcription of quantitatively normalized total RNA samples of cucumber leaves. Using cucumber genomic DNA as PCR template, fragments of all four genes were successfully amplified. However, only the SKn type *CsDHN3* gene could be PCR amplified when cDNA samples were used as template. Amplification of a *CsDHN3* specific gene fragment is also shown in Fig. 5. As an internal standard for RT-PCR expression analysis, the *Actin3* gene was selected and studied. The *CsDHN1*, *CsDHN2* and *CsDHN4* genes were not expressed to a detectable level in the samples of our experiment (Fig. S3 of supplementary material) and therefore were not analysed further. For the *CsDHN3* gene a robust and characteristic expression pattern was observed by semi-quantitative RT-PCR, producing apparently high mRNA levels exclusively under intensive fertigation of 'Oitol' plants (Fig. 5). The expression of *CsDHN3* in 'Joker' plants was influenced

to a relatively low extent and did not show any linear tendency for fertigation-concentration-dependent changes. The results of RT-PCR were further evaluated by quantitative real time PCR. The same pattern of *CsGRI*, *CsGPXI*, *CsAPXI* and *CsDHN3* genes expression were observed by this method, confirming the responsiveness of these genes to elevated fertigation and concentrated nutrients (Fig. 6 and 7). The expression of *CsAPXI* was about four times higher when the 'Oitol' plants were treated with 1.5 × HG than 0.5× HG (Fig 7A). The *CsAPXI* expression in 'Joker' plants was also increased when the elevated fertigation concentration was applied until the 2.0 × HG treatments and slightly decreased upon 2.5× HG treatment (Fig 7A). The expression of *CsGRI*, *CsGPXI* were significantly higher (*P<0.001* (Tukey test, Bio-Rad CFX Maestro built-in Software)) in 'Joker' samples in comparison to 'Oitol' plants (Fig. 7B and 7C).

Discussion

In present day cucumber breeding hybrids are released for specific cultivation methods, such as for greenhouse or open field production. The former hybrid types generally exhibit higher growth rate by utilizing a constant supply of concentrated fertigation. Open field cultivars at the same time exhibit slower growth, but are more tolerant to environmental stresses, such as cold and water shortage. Our experiments aimed at preliminary assessment of molecular and genetic factors that may be relevant for the above differences in growth and nutritional status. In the experiments 'Joker' and 'Oitol', two commercially grown, contrasting F1 hybrids were used which are cultivated usually in greenhouse and open field respectively. At the basal fertigation level (0.5 x HG) growth of 'Joker' plants was found significantly slower than 'Oitol'. Reactive oxygen species (ROS) are frequently generated upon abiotic stresses in plants reviewed by You and Chan [37], generally coupled to antioxidant responses of variable intensity. ROS can cause

cellular damage at high concentrations but the moderate level of ROS can participate in signal transduction to activate stress-tolerance mechanisms [38-41].

Ferric reducing antioxidant power (FRAP) values are indicative of antioxidants content present in plants which can deactivate radicals with oxidative effects. Malondialdehyde (MDA) is produced during lipid peroxidation and is often used as a marker for oxidative stress in plants [42,43]. Lower MDA content indicates less oxidative damage, and was described as a mark of more efficient stress tolerance by Huang et al., [44] for cucumber plants grafted on salinity tolerant rootstocks. Low MDA content has also been found to be associated with better performance under salt stress in connection with tissue-specific tolerance mechanisms in 10 Cucurbita genotypes, distinguishing such closely related species [45].

In our experiments, at basal nutrient supply antioxidant capacity was substantially lower, while lipid peroxidation was significantly higher in 'Joker' than in 'Oitol' plants. Low antioxidant capacity of 'Joker' may contribute to its observed vulnerability to oxidative stress.

In antioxidant defence *APX* genes are believed to play key roles in the ascorbate-glutathione cycle which is known to be an important H_2O_2 detoxification mechanism in planta [46]. Upregulation of *APX* and *GPX* gene expression and their activities have been reported upon different abiotic stresses [47-49]. The elimination of H_2O_2 and water formation is facilitated by APXs that reduce ascorbate as a specific electron donor [47]. Glutathione reductase enzyme (GR) regenerates GSH from oxidized glutathione (GSSG) in a NADPH-dependent manner [50]. A significant upregulation of GR was also reported when chilling stress was applied on cucumber seedlings, again indicating the role of this enzyme in stress tolerance [51]. Expression of *APX*, *GPX* and *GR* genes was found substantially higher in 'Joker' plants than in 'Oitol' at

basal nutrition level. This indicates stronger oxidative stress experienced in the former hybrid, which could be a factor leading to the slow growth rate of these plants.

At higher HG nutrition levels (1.0, 1.5, 2.0 and 2.5 × HG) no significant increase of growth occurred in either genotype. This indicates that either the maximal growth potential of both hybrids was achieved, or/and nutrient toxicity prevented further growth increase. A nominal but not significant decrease in total leaf area was observed at the highest Hoagland concentration applied, especially in case of 'Oitol'. This may become more pronounced during an extended growth period and this trend could probably culminate into a significant setback of growth and yield potentially in both hybrids. Molecular results presented suggest that nutrient derived oxidative stress indeed occurred at and above the basal fertigation level in the hybrids. Prior research were conducted to optimize and formulate the content and composition of fertigation solutions based on plant growth rate and morphological features [52,53]. The application of slightly increased level (125%) of generally recommended fertilization supply was reported to cause significantly higher fruit setting rate, number of fruits per plant and total fruit yield of cucumber cultivars grown in greenhouse [54]. Apart from differences in cultivars, fertigation regimes and growth conditions, in our experiments nutrient concentrations were increased more drastically, as well as plants were not grown till fruiting; therefore a direct comparison with these results is not feasible. A decline in overall size of barley, wheat and clover were reported for the plants grown with a supply of concentrated macronutrients compared to those supplied with normal strength of nutrient solution [55]. This might be due to an osmotic effect of the applied nutrients, resulting in low external water potential, retarding water and nutrient uptake and hence reduced growth [56]. The relative contribution of the osmotic factor and ion toxicity to the oxidative effects detected in our experimental system remains to be determined.

In horticultural practice open field cucumber hybrids usually possess higher tolerance towards environmental stresses than those grown in greenhouse [57]. At the same time, greenhouse grown hybrids (like 'Oitol') are adapted to a regular supply of highly concentrated nutrients (often delivered in soilless culture), to support fast growth. Due to efficient breeding efforts mineral assimilation capacity of current greenhouse type hybrids are generally high, while slow growing (field) genotypes may be experiencing osmotic and oxidative stress under elevated nutrition level. This effect may be due to several specific reasons. Potentially, cells of rapidly growing tissues may incorporate minerals more actively; hence display higher osmotic potential, while tissues growing slowly may sequester more unused salts in vacuoles, creating lower osmotic potential. Due to slow growth, ions are also less diluted in expanding vacuoles which may contribute to their concentration. Indeed, lower osmotic potential values were found in slow growing 'Joker' tissues, than in 'Oitol'. It should be noted however, that intensity of nutrient uptake and translocation may also be variable, representing additional factors to establish osmotic relations. Adjustment by organic osmolytes may also contribute to sinking Ψs of the tissues investigated. These possibilities need to be further tested experimentally.

Fertigation strength-dependent changes of oxidative stress responses display the same trend in both hybrids. Higher level of antioxidant/reducing power (FRAP values) and lipid peroxidation (MDA) were generally associated with more concentrated Hoagland nutrition. While antioxidant capacity was lower, peroxidation level was higher, at all corresponding nutrient supply in 'Joker' and 'Oitol' plants. With low level of free and available antioxidants (low FRAP values) 'Joker' apparently could hardly cope with pro-oxidants arising due to excess nutrition. Deeper osmotic potential in 'Joker' plants may be evoked for a potential explanation for the more strained oxidative status of this hybrid. Low osmotic potential *per se*, and potentially also ion toxic

effects may provoke excessive oxidative burden in 'Joker'. From among the minerals supplied in Hoagland solution several candidates emerge as ion specific factors, potentially contributing to the observed oxidative effect. Nitrate for example, the sole nitrogen source supplied here, was reported to induce oxidative stress on plants in several studies [58-63]. Excessive nitrogen supply reduced the activity of antioxidant enzymes in wheat, where accumulation of reactive oxygen species (ROS) and malondialdehyde increased [2]. Results however cannot be directly compared, because of unrelated plant species and experimental setup, including different nitrogen sources (urea in [2] vs. primarily Ca $(NO_3)_2$ in our experiments). In the studied 'Joker' and 'Oitol' cucumber cultivars all investigated antioxidant genes followed the same increasing trend of expression at higher HG concentrations. Genes had higher transcript levels in 'Joker' at all corresponding treatments. This is supported by the general understanding of the role and upregulation of the ascorbate-glutathione cycle and increasing GR expression under oxidative stress conditions. It also illustrates the differential behaviour of the two hybrids in this respect.

Generation of endogenous biological chemiluminescence and the underlying processes are not entirely understood, but it is commonly believed that the enhanced luminescence ensuing upon stress is mainly due to lipid peroxidation; an assumption based on the observed similarities in the dynamics of signal intensities measured in plants vs. *in vitro* oxidized lipids [64]. Representative photos from cucumber plants of each treatment that are presented alongside and in contrast with the images obtained by the NightShade LB 985 system (Fig. 4) clearly revealed the different level of oxidative stress upon elevated nutrients level. These changes correspond well with results of MDA measurements, with 'Joker' hybrid suffering stronger oxidative stress at higher fertigation levels, as indicated by both methods.

The roles of plant's dehydrin proteins in different abiotic stress tolerance mechanisms are now evident. Analysis of dehydrin genes revealed organ specificity, contrasting inducibility and distinct expression pattern upon different abiotic stresses even among members of the same DHNs class [11]. Among the four identified dehydrin genes of cucumber for *CsDHN4* low level of expression was reported in leaves of cv. Chinese long No. 9930 [36]. In line with a generally ascribed role for this class of proteins in osmotic responses, *DHN2* was highly inducible by water stress, while transcription of *DHN3* was moderately increased by desiccation [57]. At the same time *DHN2* and *DHN3* were highly and only marginally responsive to external ABA treatment, respectively. In comparison with these earlier results hybrid specific, nutrient stress induced expression of *CsDHN3* is intriguing. Based on corresponding results on lipid peroxidation, it is tempting to speculate that a role of *CsDHN3* may be in redox protection, especially against lipid peroxidative damage of membranes. The involvement of dehydrins in redox protection is well known in the literature [22,65]. We conclude that *CsDHN3* gene probably contributes to redox protection against nutrition triggered lipid peroxidative stress in the 'Oitol' hybrid. Lack of this protection probably coupled with low antioxidant levels predisposes 'Joker' plants to high oxidative strain. This assumption is supported by sequence features of *CsDHN3*. It belongs to the SKn-type dehydrins, with proposed protective functions by membrane binding. Peroxidation of membrane lipids can be counteracted by expressed *CsDHN3* proteins acting as molecular chaperones or ion sequestration agents [66]. Free radical scavenging activity of the SKn-type *SbDHN2* has been also observed and suggested to be linked to the high percentage of glycine and histidine residues present in this type of *DHN*s [67].

Conclusions

The presented experiments aimed at substantiating physiological factors that may underlie the contrasting behaviour of open field and greenhouse cultivars, and clarifying molecular biological differences, especially those relevant for nutritional effects. In these studies a fertigation gradient with a maximum of 2.5 × Hoagland nutrition level was applied to two representative F1 cultivar hybrids (‘Oitol’ bred for greenhouse and ‘Joker’ for open field). Experimental data allow several conclusions to be drawn: High nutrition level may create osmotic stress and oxidative burden in cucumber. This effect was found hybrid specific. In our case a slow growing, field cultivated hybrid (‘Joker’) suffered higher osmotic stress and oxidative damage than a hybrid intended for greenhouse production (‘Oitol’). *CsDHN3*, a member of the dehydrin gene family in cucumber, displayed specific induction under high nutrition in ‘Oitol’ hybrid only. This suggests a unique role of this protein in protecting plants from the nutrient induced oxidative damage detected in ‘Joker’. Bio photon emission imaging was successfully used to confirm biochemical data about oxidative damage in the leaves. This method therefore was assured as a non-invasive, high-throughput tool, capable of fast characterization of oxidative stress level in cucumber plants under different nutritional regimes.

Our results reveal contrasting physiological responses and some distinctive molecular features in cucumber F1 cultivar hybrids under standard and high fertigation levels.

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Conflicts of Interest

The authors declare no conflict of interest.

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Table 1

Target genes, oligonucleotide primers and expected product sizes in RT-PCRs and RT-qPCRs

Gene	Accession No	Forward primer (5′-3′)	Reverse primer (5′-3′)	Amplicon size (bp)
CsaDHN1	XM_011656037.1	CTATCCAATTCGCCAGACCG	GATGTCCACTCCCATCCTCT	191
CsaDHN2	XM_011654848	ATGGTGGCATACTTCATCGC	CAAGCAGTGTCAACCACGAC	154
CsaDHN3	XM_011659051	GAGAAACTCACCCGATCC	TCCTTCTTTTCCTCTTCTCC	146
CsaDHN4	XM_004150027	TGATGGACAAGGCGGGAGG	GCATCACGAAAGCACCACC	124
CsaAPX1	XM_004149001	TCACACATTGGGTAGGGCA	TATGCTGCCGATGAGGATG	203
CsaGPX1	XM_004145445.3	ATCAAGTGCTGGAGGGTTT	GATGTTGTTGGTGGGTATCTC	105
CsaGR1	XM_011652579.2	TACGATCTCTGGCCGACAAGAG	ATGGGTGTATTCCAACAGTGCTG	182
CsaAct-3	DQ115883	GGCAGTGGTGGTGAACATG	GACTCACACCATCACCAGAA	151

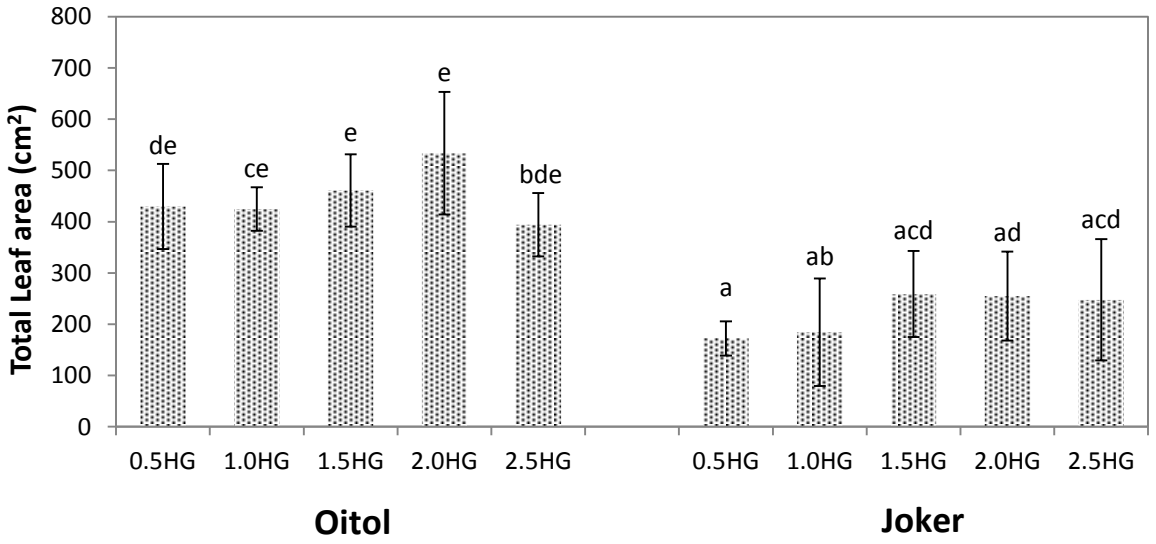

Fig. 1. Total leaf area of two F1 cucumber cultivar hybrids ('Oitol' & 'Joker') treated with different strength of fertigation solution. Different letters denote significantly different values according to Shapiro-Wilk's test (p<0.05). Error bars represent standard deviation of the mean among at least three biological replicates.

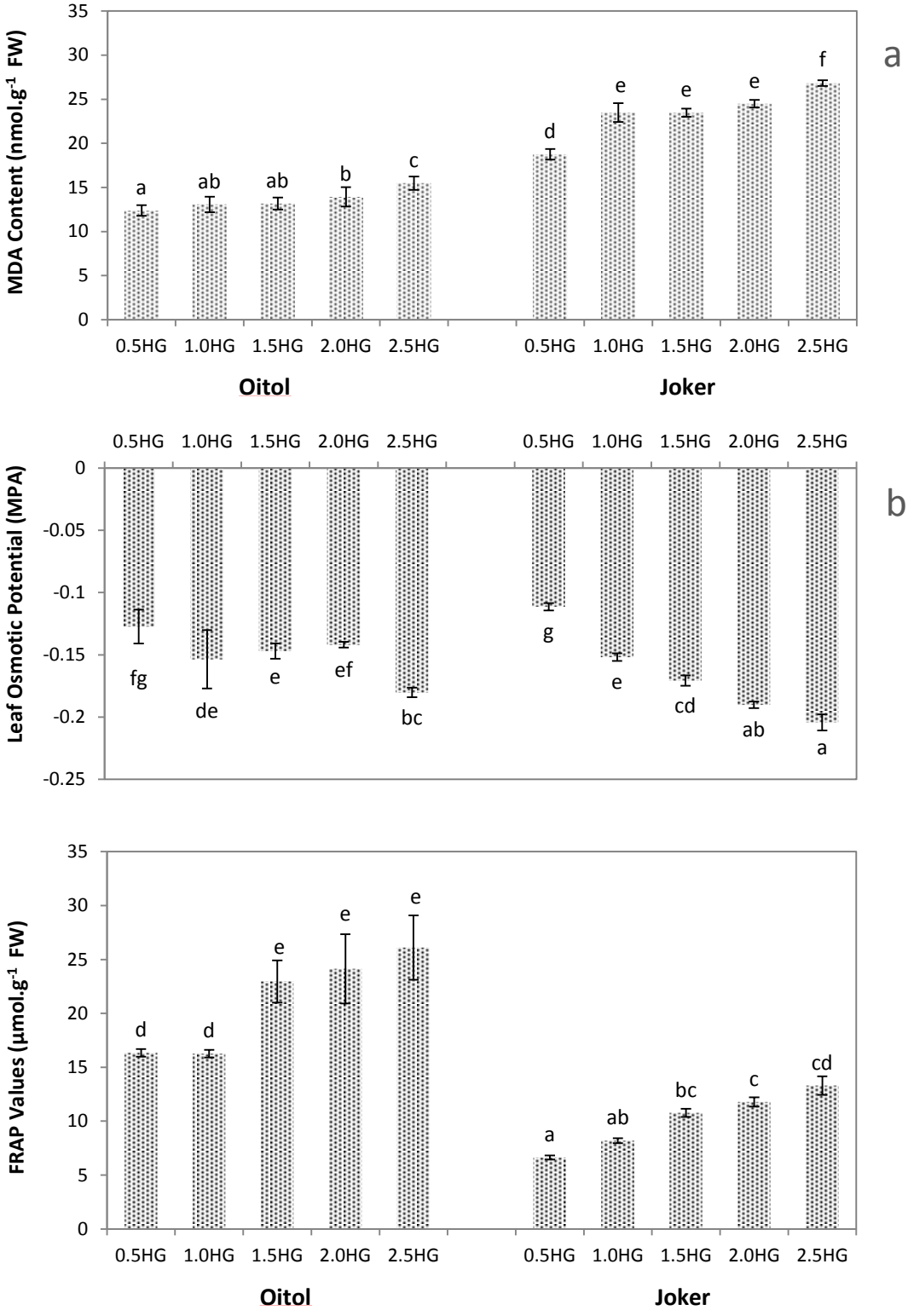
MDA Content (nmol.g-1 FW)
35
30
25
20
15
10
5
0
a
ab
ab
b
c
d
e
e
e
f
0.5HG
1.0HG
1.5HG
2.0HG
2.5HG
Oitol
Joker
a
Leaf Osmotic Potential (MPA)
0
-0.05
-0.1
-0.15
-0.2
-0.25
fg
de
e
ef
bc
g
e
cd
ab
a
b
FRAP Values (µmol.g-1 FW)
d
d
e
e
e
a
ab
bc
c
cd

Fig. 2. [a] Malondialdehyde concentration in leaves, [b] Leaf osmotic potential, and [c] Antioxidant capacity (FRAP values) of two F1 hybrid cultivars ('Oitol' & 'Joker') treated with various strength of fertigation solutions. Different letters are for significantly different values according to the Shapiro-Wilk's test (p<0.05). Error bars represent standard deviation of the mean among three biological replicates.

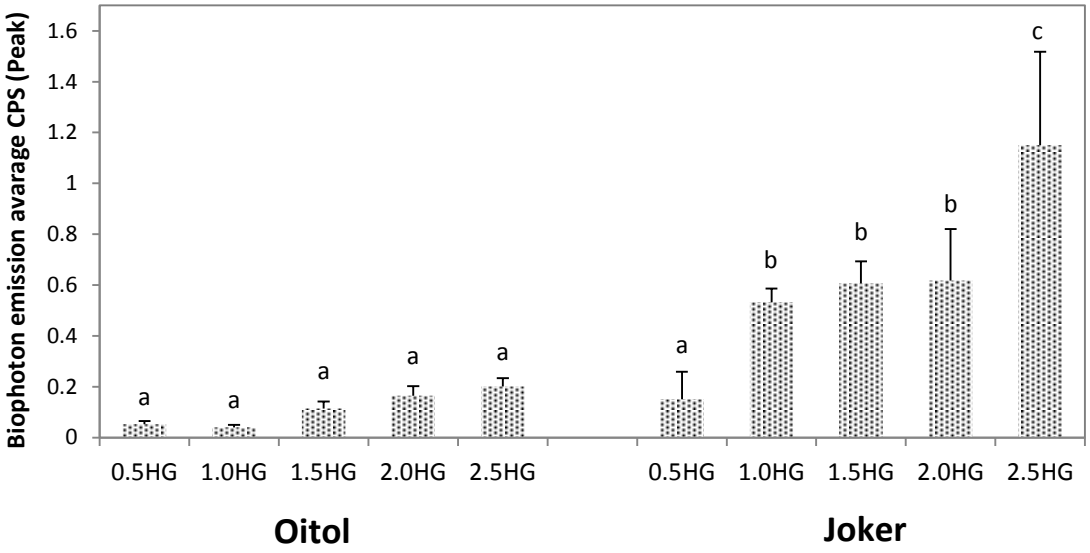

Fig. 3. Results of biophoton emission (count per second) recorded from leaves of cucumber hybrids ('Oitol' & 'Joker') treated with different strength of fertigation solution. Different letters are for significantly different values according to Shapiro-Wilk's test (p<0.05). Error bars represent standard deviation of the mean among three biological replicates.

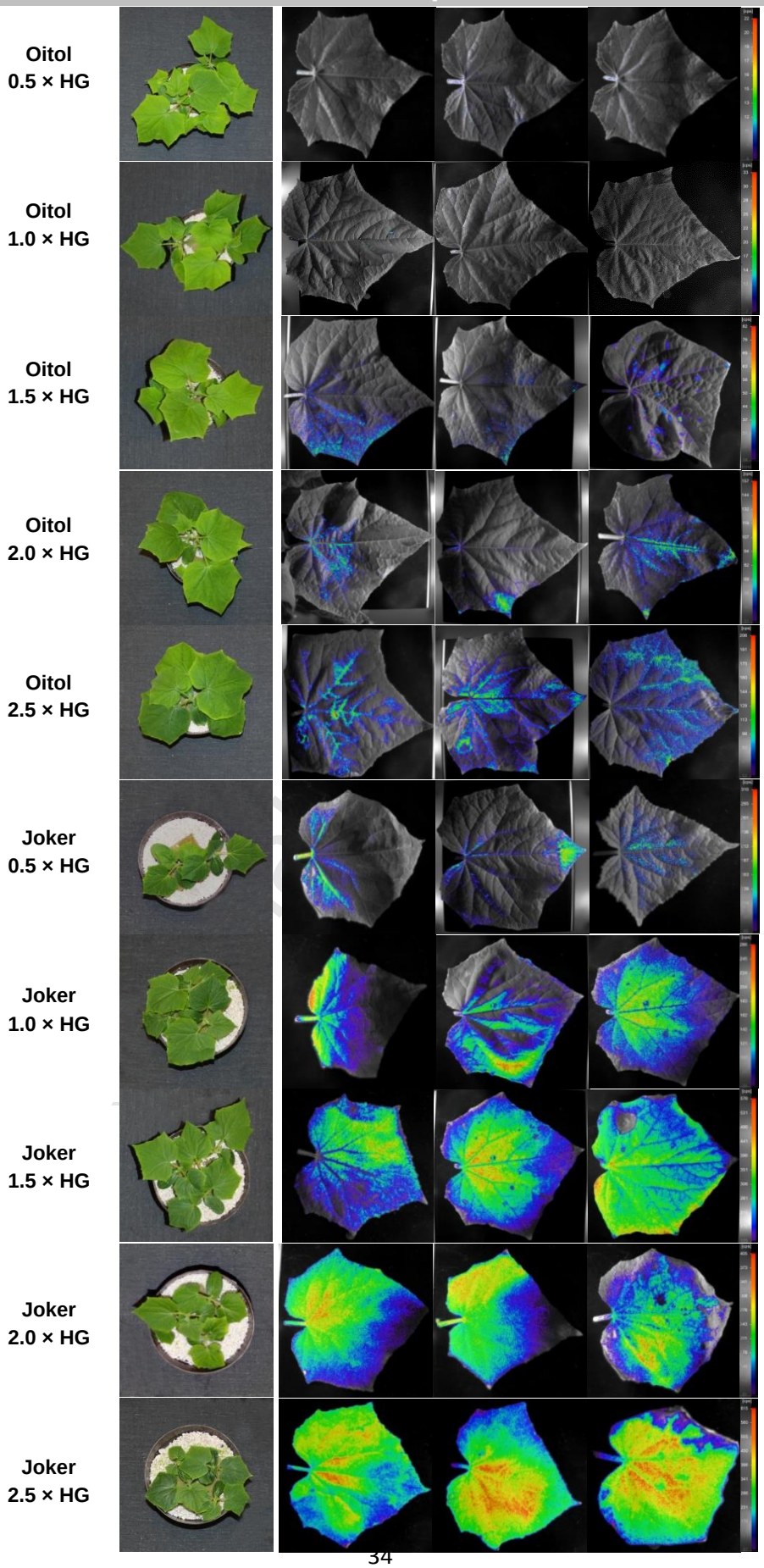
Oitol
0.5 × HG
Oitol
1.0 × HG
Oitol
1.5 × HG
Oitol
2.0 × HG
Oitol
2.5 × HG
Joker
0.5 × HG
Joker
1.0 × HG
Joker
1.5 × HG
Joker
2.0 × HG
Joker
2.5 × HG

Fig. 4. Visualised biophoton emission recorded from leaves of two cucumber F1 cultivar hybrids ('Oitol' & 'Joker') treated with different strength of Hoagland fertigation solution. The photocount-comparison is visually depicted by pixel peak-distributions on 2 D-images represented by pseudo colour-coded pixel intensity values on a 4096-scale (see relative intensity bars on the right side of the images)

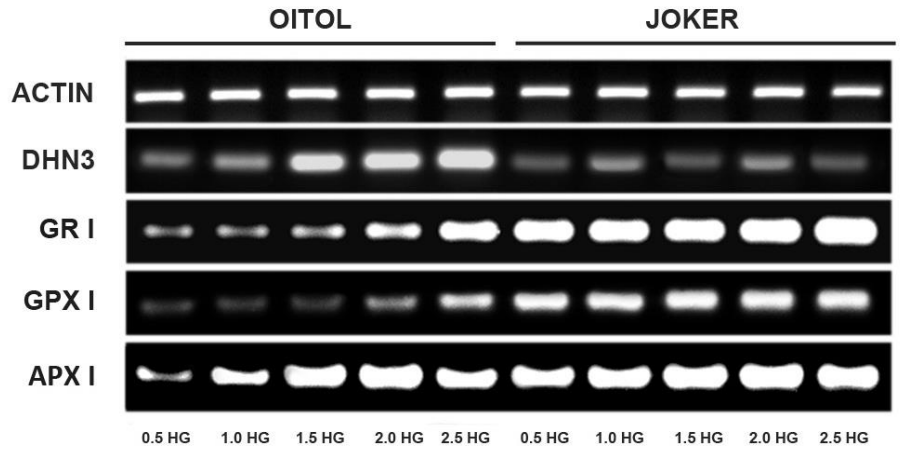

Fig. 5. Semi quantitative RT-PCR analysis of *CsDHN3, GR1, GPX1, APX1 and Actin 3* genes in two cucumber cultivar hybrids ('Oitol' & 'Joker') under gradient increase of Hoagland fertigation level (× HG).

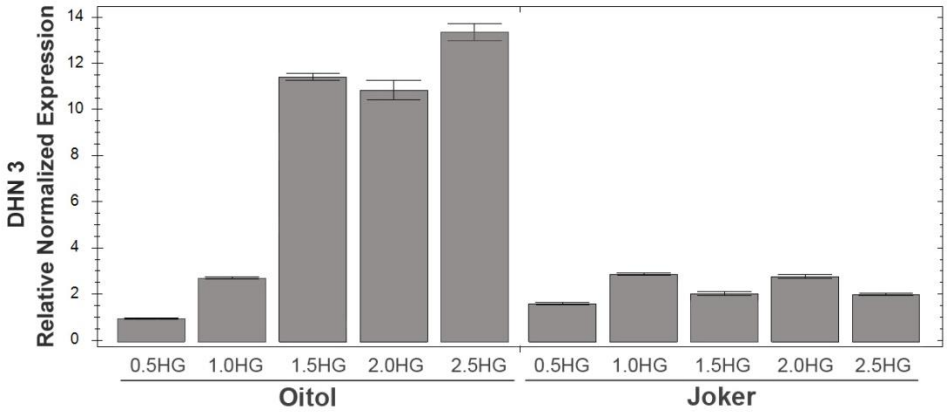

Fig. 6. Relative normalized expression of *DHN3* gene in two cucumber cultivar hybrids ('Oitol' & 'Joker') under gradient increase of Hoagland fertigation level (× HG). Error bars represent standard deviation of the mean among three biological replicates.

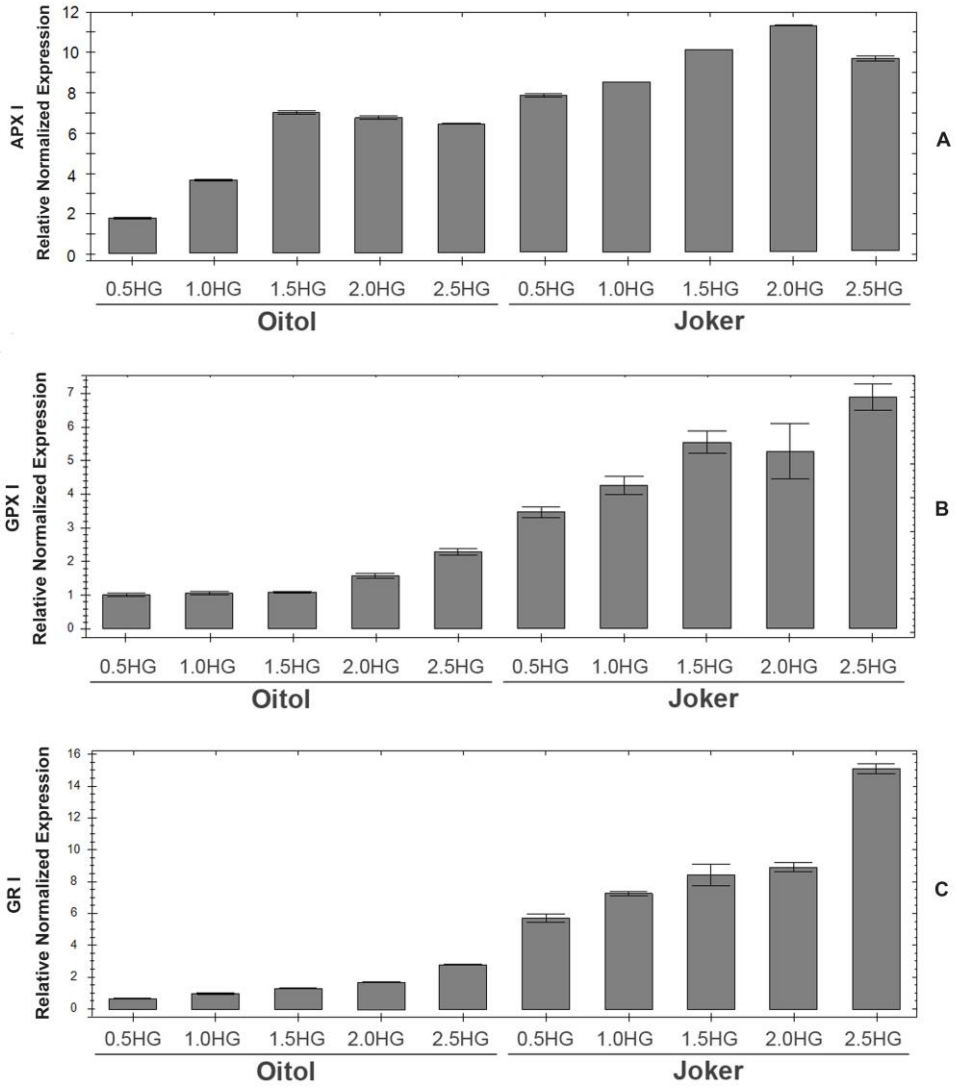

Fig. 7. Relative normalized expression of *CsAPX1*(A), *CsGPX1*(B), and *CsGR1*(C), in two cucumber cultivar hybrids ('Oitol' & 'Joker') under gradient increase of Hoagland fertigation level (× HG). n=3

Author's contributions

I.M., A.S. and I.P designed the research, R.O., I. M., O.B., S.J and Z.P. performed the experiments; R.O., A.S. and I.M., analysed the data; I.M. and I.P interpreted the results and wrote the manuscript, with contribution and approval from all authors.