Reduced light and moderate water deficiency sustain nitrogen assimilation and sucrose degradation at low temperature in durum wheat

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SUMMARY

The rate of carbon and nitrogen assimilation is highly sensitive to stress factors, such as low temperature and drought. Little is known about the role of light in the simultaneous effect of cold and drought. The present study thus focused on the combined effect of mild water deficiency and different light intensities during the early cold hardening in durum wheat (*Triticum turgidum ssp. durum* L.) cultivars with different levels of cold sensitivity.
The results showed that reduced illumination decreased the undesirable effects of photoinhibition in the case of net photosynthesis and nitrate reduction, which may help to sustain these processes at low temperature. Mild water deficiency also had a slight positive effect on the effective quantum efficiency of PSII and the nitrate reductase activity in the cold. Glutamine synthesis was affected by light rather than by water deprivation during cold stress. The invertase activity increased to a greater extent by water deprivation, but an increase in illumination also had a facilitating effect on this enzyme. This suggests that both moderate water deficiency and light have an influence on nitrogen metabolism and sucrose degradation during cold hardening. A possible rise in the soluble sugar content caused by the invertase may compensate for the decline in photosynthetic carbon assimilation indicated by the decrease in net photosynthesis. The changes in the osmotic potential can be also correlated to the enhanced level of invertase activity. Both of them were regulated by light at normal water supply, but not at water deprivation in the cold. However, changes in the metabolic enzyme activities and osmotic adjustment could not be directly contributed to the different levels of cold tolerance of the cultivars in the early acclimation period.

Keywords:

Cold; drought; durum wheat; invertase; light; nitrogen assimilation

Abbreviations:

Abs, absorptivity of incident light; D, mild water deficiency; ETR, apparent electron transport rate; Fv/Fm, maximal quantum efficiency of PSII; gs, stomatal conductance; GS, glutamine synthetase; I, normal irrigation; LL, low (reduced) light; NIR, near infrared light; NL, normal light; NPQ, nonphotochemical quenching parameter; NR, nitrate reductase; PAR, photosynthetically active radiation; Pn, net photosynthesis; PPFD, photosynthetic photon flux density; PSII, photosystem II; R, red light; ROS, reactive oxygen species; RWC, relative water content; Y(II), effective quantum yield of PSII

Introduction

In areas with a continental climate crops are exposed to very fluctuating weather conditions. Low temperature and water deficiency adversely affect the growth and development of cereal crops. These conditions are frequently experienced in late autumn if preceded by a dry summer. These two stressors have a prominent impact on the annual yield.

Low temperature induces a wide range of physiological and biochemical responses. Membrane rearrangement causes changes in the stability and mobility of proteins and a shift in redox poise which decreases enzyme activities. Changes in membrane structure and the additional effect of light energy often act as stress factors and cause an imbalance in light utilization compared to optimal temperature conditions (Ruelland et al., 2009). At low temperature, the overexcitation of the photosystems induces photoinhibitory conditions, which results in the accumulation of reduced components in the photosystems, a decrease in energy-containing substances such as ATP, and the build-up of free radicals (ROS), which degrades the light-harvesting complexes (Keren and Krieger-Liszkay, 2011). High levels of ROS inhibit the activity of RuBisCO and fructose-1,6-bisphosphatase (Hurry et al., 1994). Further, low temperature temporarily inhibits sucrose synthesis, which contributes to the depletion of ATP reserves via a low level of inorganic phosphate. The deceleration of ATP synthesis and the regeneration of ribulose-1,5-bisphosphate can be attributed to the limited capacity for CO_2 fixation and the consequent decline in photosynthetic sugar synthesis (Ensminger et al., 2006).

Low temperature limits net photosynthetic activity via photoinhibition, while water deprivation primarily induces stomatal closure, which also reduces the intercellular CO_2 concentration. The RuBisCO activity is also known to be affected by drought (Reddy et al., 2004). Thus, low temperature and water deficit may greatly impede sugar synthesis in the Calvin cycle, which triggers other metabolic pathways to compensate the sugar deficit and provide reducing agents for assimilation. One of these responses is the remobilisation of sugars from carbohydrate reserves (Gupta and Kaur, 2005).

Besides CO_2 fixation and sugar synthesis, the uptake of nitrate and the incorporation of NH_4^+ into organic compounds are also important for adequate plant growth and development. The initial step, namely the conversion of inorganic nitrate to nitrite is catalysed by the enzyme nitrate reductase (NR). A further enzymatic reaction, the synthesis of glutamine catalysed by glutamine synthetase (GS), is pivotal in the conversion of inorganic ammonium to organic nitrogenous compounds, e.g. amino acids. Both NR (Campbell, 1999) and GS reaction greatly depends on light which directly modulates the GS activity in leaves at transcriptional (Oliveira and Coruzzi, 1999) via photorepector-mediated way and post-translational level via phosphorylation of the GS protein (Lima et al., 2006). The efficiency of photosynthesis is light-dependent and provide ATP and the carbon skeleton for amino acid synthesis. Beside light, the abundance of GS and RuBisCO showed a positive correlation with the severity of stress, including low temperature (Simonović and Anderson, 2007) and drought (Bernard et al., 2008; Nagy et al., 2013).

When CO_2 fixation and sugar synthesis in the Calvin cycle are disturbed, the deficit of sugar supply impedes numerous biochemical reactions which require sugars as substrate, such as glutamine synthesis. The remobilisation of soluble sugars is widely regarded as an adaptive response to limited CO_2 availability or to osmotic stress, when there is a considerable shift in the sink/source relationship in the carbohydrate metabolism (Gupta and Kaur, 2005). Higher sucrose content was reported in several monocot species in the case of water deficit (Kaur et al., 2007; Fu et al., 2010). Both cold and drought greatly promote starch degradation, and in turn the synthesis of sucrose (Rosa et al., 2009). Beta-fructofuranosidase (invertase) has several isoforms which catalyse the conversion of sucrose to glucose and fructose. The

activity of this enzyme is induced by drought stress and regulated by light in several plant species (Koch, 2004; Fotopoulos, 2005; Rabot et al., 2012). The separate effects of drought and cold on invertase activity have been described previously; however, less is known about their combined effect. However, the regulation of invertase activity by light is still not clearly described.

Durum wheat is an important cereal, numerous cultivars of which are adapted to drought (Araus et al., 2002). Previous studies demonstrated that stomatal conductance, the transpiration rate, growth and yield were less affected in durum wheat than in bread wheat during water deficiency (Moayedi et al., 2010). In addition, differences in the nutrient uptake and distribution were also described in bread and durum wheat under drought conditions (Zubaidi et al., 1999), suggesting differences in their drought tolerance.

The separate effects of water deficit and low temperature on carbon and nitrogen metabolic processes have been well characterized, whereas the simultaneous effect of these two abiotic stress factors is less known (Mittler, 2006). It is well documented that cold hardening and the light intensity during the cold phase have pivotal roles in the acclimation and development of frost hardiness. However, this strongly depends on the length and severity of the stress (Gray et al., 1997; Janda et al., 2014). It is still not clear how water deprivation and light intensity affect the efficiency of carbon and nitrogen assimilation, and sucrose degradation under cold conditions.

Considering previous findings, an attempt was made in a short-term cold-hardening experiment to explore how light intensity modifies the photosynthetic and nitrogen assimilation rate in durum wheat lines with different levels of cold sensitivity at low temperature, to reveal whether moderate water stress improves the efficiency of these processes at low temperature, to detect the changes in the sucrose degradation and osmotic adjustment of cells in response to the treatments and find the possible relationship between the parameters and the cold-tolerance. In addition, the length of sufficient hardening time will be discussed.

Materials and methods

Growth conditions

Two winter durum wheat (*Triticum turgidum ssp. durum* L.) cultivars (Mv Makaróni, MvTD10-10) with contrasting cold tolerance were used in the experiments. In preliminary experiments, Mv Makaróni was found to be cold-tolerant and MvTD10-10 to be cold-sensitive on the basis of 3-year freezing survival tests (unpublished data). In the current experiment, the plants were grown in pots (15 seeds/pot) filled with loamy soil and sand (3:1 v:v) for 21 d in a Conviron PGR-36 growth chamber (Controlled Environment Ltd, Winnipeg, Canada). The temperature was at 21°C, 75% relative humidity, a 16/8 h photoperiod and PPFD=250 μmol m^{-2} s^{-1} (normal light, NL). After 10 d of sowing, the 50 pots of each genotype were divided into two groups, the first of which continued to grow with normal water supplies [35±5% relative soil humidity (I)], while the second was subjected to mild water deficiency [15±5% relative soil humidity (D)]. Soil humidity was checked daily using a soil moisture meter with an SM200 sensor (Delta-T Devices, Cambridge, UK).

Three-week-old plants grown under either I or D conditions were transferred to low temperature (5°C) for 2 weeks under normal (NL) or low light (LL, PPFD=20 μmol m^{-2} s^{-1}) conditions. Measurements on relative water content (RWC), gas exchange and chlorophyll-*a* fluorescence and the collection of samples for enzyme assays were performed on the 3-week-old plants and at the end of the cold hardening period (on 5-week-old plants).

Determination of the RWC content and osmotic potential

The RWC of the plants was determined from 0.2 g FW of leaves of 5 plants from each treatment and calculated as RWC % = (FW-DW) (SW-DW)$^{-1}$ x 100, where FW is the fresh weight, SW the water-saturated weight and DW the oven-dried weight (80°C for 48 h). The FW and DW data were also used as a correction factor in the calculation of enzyme activities.

The osmotic potential of the leaf sap was determined by using an Osmomat 030, freezing-point osmometer (Gonotech, GmbH, Berlin, Germany). Leaves sap was obtained after frozen of leaves in liquid nitrogen, thawing, exposed by Tissue lyser (QIAGEN) and centrifuged at 13000 g for 10 min. The leaf saps were kept at -10°C until their freezing-points were determined.

Gas exchange analysis

The gas exchange analysis was performed on the third fully developed leaves with a Ciras 2 Portable Photosynthesis System (Amesbury, USA) at a common laboratory temperature using a narrow leaf area (2.5 cm^2) chamber. The CO_2 assimilation rate (Pn) and stomatal conductance (gs) parameters were determined at the steady-state level of photosynthesis using a CO_2 level of 380 ppm and light intensity of 250 μmol m^{-2} s^{-1}.

Chlorophyll a fluorescence induction

Measurements of chlorophyll *a* fluorescence induction were carried out at a common laboratory temperature using a pulse amplitude modulated fluorometer (Imaging-PAM M-Series fluorometer; Walz, Effeltrich, Germany). The plants were dark-adapted for 30 min before the photosynthesis was activated using 270 μmol m^{-2} s^{-1} light intensity (PAR). A 0.8 s saturation pulse (PPFD=3000 μmol m^{-2} s^{-1}) provided by a blue LED-Array Illumination Unit IMAG-MAX/L (λ=450 nm) was applied for determination of the Fv/Fm,Y(II), ETR and NPQ parameters. The Y(II) was determined under steady state conditions according to the nomenclature described by Klughammer and Schreiber (2008). NPQ was calculated as (Fm-Fm')/Fm' and ETR = Y(II) x PAR x 0.5 x Abs, where Abs. was determined based on Abs = 1 – R/NIR.

In vivo enzyme assay of the NR (EC 1.6.6.1)

The method was an adaptation of the in vivo NR assay of Pécsváradi and Zsoldos (1996). One hundred mg of leaves were cut into 5 mm sections and incubated in 2 mL of 100 mM K-phosphate buffer (pH 7.5) containing 25mM KNO_3 and 4% n-propanol) at 30 °C in the dark for 2 h. They were then boiled at 100 °C for 5 min to stop the conversion of NO_3^- to NO_2^-. The nitrite production was detected colorimetrically by adding 500 μL 0.3% 1-naphthylamine and 500 μL 1% sulphanilamide (dissolved in 30% acetic acid) to the reaction mixture. After 15 min, the optical density of solutions was measured at 540 nm. The incubation buffer was used as blank. The NR activity was calculated in nkat units g^{-1} DW using a standard curve based on known nitrite dilutions.

Enzyme assay of the GS (EC 6.3.1.2)

The activity of the GS enzyme was determined in vitro with a modified version of the 'synthetase reaction', by measuring the formation of γ-glutamyl monohydroxamate (Pécsváradi et al., 2009). Five hundred mg of leaf samples were finely ground in a mortar in 2

mL of extraction buffer [0.2 M TRIS-HCl (pH 7.5) containing 9.6% glycerol, 2 mM EDTA, 3 mM DTT, 1 mM GSH], after which the extracts were centrifuged at 10 000 g for 10 min. Following this, 100 µL of supernatant was mixed with 250 µL of reaction buffer containing 50 mM imidazole, 18 mM ATP-Na_2, 28 mM $MgCl_2$, 25 mM hydroxylamine and 92 mM L-glutamate-Na (pH 7.2) and the reaction mixture was incubated at 30 ^oC in a water bath for 20 min. The enzyme reaction was terminated by adding 500 µL of stopping solution (370 mM $FeCl_3$, 200 mM TCA, 700 mM HCl). After centrifugation (5 min, 13 000 g) 600 µL of supernatant was mixed with 300 µl of water in a semi-micro cuvette. The GS activity was measured at 540 nm against an immediately stopped (0 min) parallel sample, and was calculated in nkat units g^{-1} DW using a standard curve based on known dilutions of γ-glutamyl monohydroxamate.

Enzyme assay of invertase (β-D-fructofuranoside fructohydrolase; EC 3.2.1.26)

Five hundred mg of leaf samples were finely ground in a mortar in 1 mL 100 mM sodium acetate buffer (pH 4.5) at 55ºC and then centrifuged at 10 000 g for 10 min, after which 0.5 mL 3 mM sucrose was added to 0.5 mL supernatant and the reaction mixture was incubated at 55 °C for 20 min. The reaction was stopped at 95°C, after which 1.9 mL of 3,5-dinitrosalicylic acid (DNS) reagent (containing 44 mM DNS, 1 M $NaKC_4O_6$ x $4H_2O$ in 0.35M NaOH) was added to 0.1 mL of the samples, which were incubated at 95°C for 10 min. Absorbance was measured at 540 nm after cooling. The enzyme activity was calculated in nkat g^{-1} DW units using a calibration curve (0, 300, 400, 500, 600, 750, 900 and 1000 µg mL^{-1} L-glucose), which relates the measured absorbance to an equimolar mixture of glucose and fructose.

Statistical analysis

The results were obtained from at least five biological replicates. Differences between treatments or genotypes within each treatment were determined by means of Tukey's post hoc test (P<0.05) using the SPSS 16.0 software.

Results

Cold-induced changes in the initial steps of the carbon and nitrogen assimilation processes were investigated in two winter durum wheat cultivars with different levels of frost tolerance exposed to two water regimes and two light levels. Water deprivation was begun when the plants were 10 d old and continued until the end of the experiment.

Changes in the RWC

Mild water stress induced a similar decrease in RWC in both cultivars at both temperatures. When the plants with normal water supply were transferred to low temperature, the RWC values did not change significantly. Light intensity did not affect the RWC content of the leaves. The RWC values, however, were significantly higher in drought-stressed MvTD10-10 plants under both illumination (NL, LL) at 5°C (Table 1).

Changes in the CO_2 assimilation (Pn) and stomatal conductance (gs)

In response to water deficit, Pn decreased in both cultivars at the control temperature (21°C), especially in the case of MvTD10-10 plants. Cold hardening induced a significant

decrease in Pn both in water-stressed and irrigated plants. However, higher Pn was observed in plants grown at LL than at NL, suggesting a facilitating effect of LL on photosynthtetic carbon assimilation in both irrigation regimes. The difference in Pn between NL and LL was more pronounced when water deprivation occurred at 5°C (Fig. 1A). The difference between the cultivars was greater at control temperature (21°C) and diminished at low temperature, irrespective of the water conditions. Changes in the gs values were similar to those in Pn (Fig. 1B).

Fv/Fm, Y(II), ETR and NPQ

The Fv/Fm ranged between 0.7 and 0.79 during the experiments. Water deprivation did not affect Fv/Fm. Low temperature induced a small decrease (10%>) in Fv/Fm in the case of NL. Changes were irrespective of the cultivars (Fig. 2A).

The Y(II) was also determined under steady state conditions after illumination with 270 μmol m^{-2} s^{-1} light intensity. At the control temperature (21°C), water deficiency induced a decrease in Mv Makaróni, while slightly higher Y(II) was observed in the case of MvTD10-10. Low temperature (5°C) caused a significant decrease in Y(II) in irrigated plants, while the temperature-induced decrease was less pronounced under water deprivation. The different growth light conditions (i.e. NL, LL) did not modify the light-induced photosynthetic efficiency of PSII (Fig. 2B).

The changes in the ETR showed similar pattern to Y(II), however, the effect of light was more pronounced in Mv Makaróni at both water regimes. Absorptivity of incident light showed a non-significant fluctuation between the treatments (Fig. 2C). Nonphotochemical quenching (NPQ) parameter decreased only in the MvTD10-10 of the effect of water loss at 21°C. Cold-hardening under normal irrigation induced a slight statistically not significant elevation in NPQ. Water stressed plants showed significantly lower NPQ values compared to the normal irrigated plants at low temperature. Neither light nor genotype effects were found in the NPQ between the treatments (Fig. 2D).

Differences in the NR and GS activity

The NR activity decreased significantly at the control temperature (21°C) in response to water deficit, to a similar extent in both cultivars. At low temperature, reduced NR activities were detected in irrigated plants, while in drought-stressed plants a decrease was only observed in plants grown at NL. Higher NR activity was detected at LL and low temperature as compared to NL. Differences in NR activity between the cultivars were detected in irrigated plants grown at low temperature, when higher activity was detected in MvTD10-10 plants (Fig. 3A).

At the control temperature (21°C), higher GS activity was detected in genotype MvTD10-10 than in Mv Makaróni, and the GS activity declined significantly under drought stress, irrespective of the cultivar. At low temperature, the GS activity decreased in irrigated plants, and remained low during water deprivation in both cultivars. Light-dependent changes in GS activity were only detected in MvTD10-10 plants grown with normal water supplies, as the GS activity showed a higher level in the NL than in the LL treatment (Fig. 3B).

Invertase activity and osmotic potential

Invertase activity was similar in both genotypes when grown at 21°C, and increased to a similar extent during water deprivation in both genotypes. Elevated invertase activity was observed in plants grown at low temperature under both well-irrigated and water-deprived

conditions. Significantly higher invertase activity was observed in the case of water deficiency as compared to normal water supplies. In the case of MvTD10-10, higher invertase activity was detected in plants grown under NL than in those grown at LL intensity irrespective of the water conditions, but this difference was not significant in the case of Mv Makaróni (Fig. 4A).

Water deprivation caused a significant increase in the osmotic potential of leaf saps both at the control (21°C) and low (5°C) temperatures. In addition, the 2 weeks cold hardening period also elevated the osmotic potential of leaf sap in the irrigated plants grown at NL. Both light and water deprivation showed similar changes in the osmotic potential to the invertase activity during the cold hardening. No significant difference was found between the cultivars (Fig. 4B).

Discussion

Photosynthesis, nitrogen- and sucrose metabolism are strongly affected by adverse environmental factors, such as low temperature or water deficit. Mittler (2006) emphasized that combined stress is not merely an addition of two individual stresses and regarded as a novel stress. Besides other combinations of cold to other stresses, the effect of low–temperature stress to drought at different illuminations on the carbon and nitrogen assimilation has been rarely reported. In the present work, the effects of light was combined with water deprivation on photosynthetic quantum efficiency, CO_2 assimilation, and the activity of enzymes responsible for the nitrogen assimilation and sucrose remobilisation were studied in durum wheat cultivars in the early phase of cold-hardening.

Previous gene expression analysis demonstrated that NR, GS and invertase have light-dependency at the gene expression level in bread wheat during cold hardening. Changes in the gene expression suggests potential changes at metabolic level (Majláth et al., 2012). In bread wheat, mild drought at low temperature was contributed to the homeostasis of oxidative metabolism and relatively better photosynthesis, and hence to less grain yield loss under late spring low temperature stress, however, the effect of light was not studied (Li et al., 2014). Exposure of plants to low but not freezing temperature is required for the development of freezing tolerance. It has been demonstrated that hardening is much less effective in the dark than under normal light conditions (Janda et al., 2014). However, exposure of plants to low temperatures also enhances the level of photoinhibition, which does not only impair the the photosynthetic machinery, but may also modify other stress-related processes (Szalai et al., 1997). Contrary to bread wheat, Szűcs et al. (1999) observed that four to six weeks are necessary for the development of adequate cold-hardiness in durum wheat.

The results of gas exchange analysis showed that Pn was higher in plants grown under LL than under normal light NL at low temperature. This light dependency was also observed during mild water deprivation, and Pn was found to be reduced by the stomatal closure caused by drought. Photosynthesis efficiency at the level of light reactions is indicated by Fv/Fm and Y(II). Fv/Fm was not affected by water deprivation. However, when cold was combined with NL a small decrease was observed in Fv/Fm which recovered under LL. These negligible decreases in Fv/Fm show that there was no irreversible damage to PSII. Unlike Pn, the Y(II) was not affected by LL at low temperature. During the cold, Y(II) was significantly improved by the moderate water deprivation with the exception of MvTD10-10 at NL-adapted state. Light had no statistically significant effect on Y(II) under our experimental conditions. The changes in the apparent electron transport rate (ETR) showed similar pattern to the Y(II), however, the effect of light intensity was more pronounced in Mv Makaróni at both water regimes. Traditional ETR formula uses the absorptivity constant (Abs=0.84) which is a mean value for a large number of healthy green leaves. In our

experiment, Abs of incident light was determined at the individual measurements which provides more precise ETR calculation regarding the inhomogeneity in photosynthetic pigment composition, alteration in thylakoid ultrastructure as well as age and stress (Merzlyak et al., 2008). Hence, differences between the Y(II) and ETR may caused by the fluctuation in Abs. of incident light. Other authors reported changes in ΔF/Fm′, which can be correlated to Y(II) (Klughammer and Schreiber, 2008). A 20% decrease in ΔF/Fm′ was previously found during the formation of the fluorescence induction curve, while no great differences were observed between control and dehydrated durum wheat leaves under steady state conditions (Flagella et al., 1995). In the present experiment, the cold-induced drop in Y(II) recovered to the level at growth temperature (21°C) under the effect of mild water stress. It is possible that other scavenging processes, such as alternative electron flux, act to dissipate excess light energy, as also found in water-stressed wheat plants (Zivcak et al., 2013). NPQ is a good indicator of the pH-dependent heat dissipation of the excess excitation energy. In both durum cultivars, NPQ was significantly lower at drought but not at the normal watered conditions during the cold-hardening. This may demonstrate that NPQ involved in the light energy release during the combined cold and drought stress. However, this manner of the excitation energy release was independent to light.

Low temperature temporarily inhibits sucrose synthesis, which plays an important role in the inorganic phosphate supply of the cell under non-stressed conditions. A recovery in the photosynthesis and sucrose synthesis enzymes was observed following long-term cold hardening in winter rye and winter wheat (Hurry et al., 1994). The rate of sucrose synthesis may affect various assimilation processes, such as glutamine synthesis, via a decrease in ATP formation.

On the other hand, the insufficient conversion of sucrose to glucose and fructose, which is catalysed by invertase, inhibits its own enzymatic activity and may be a key limiting step at certain developmental stages in cereals during drought (Rosa et al., 2009) and cold stress (Oliver et al., 2007). Increases in invertase activity in the leaves of drought-tolerant bread wheat were also found during mild water deficiency (Saeedipour and Moradi, 2011). Higher levels of soluble sugar, such as glucose and fructose, were found in frost-tolerant Wassilewskija-2 than in frost-sensitive Cape Verde Islands-1 *Arabidopsis* ecotypes after two-weeks of cold hardening (Cook et al., 2004). In the durum cultivars Mv Makaróni and MvTD10-10, the CO_2 uptake was limited during both drought and cold, as indicated by the low Pn and stomatal conductance values. However, the sucrose hydrolysis was higher not only during the drought, but also under a normal water regime at low temperature. NL also enhanced the activity of invertase in MvTD10-10 under both water regimes, which reflects its light dependency. A higher level of sucrose hydrolysis was also found in water-stressed wheat plants under NL (Munns and Weir, 1981). In this connection, it seems that both mild water deficiency and NL are able to compensate for the reducing effect of cold on sugar remobilisation in these durum cultivars via the higher activity of invertase.

The remobilisation of sugars under stress conditions could be attributed to various metabolic pathways, including amino acid synthesis (Nunes-Nesi et al., 2010). The activity of the two key enzymes, NR and GS, provides relevant information on the overall condition of the plants. Under severe stress conditions, included cold and drought, the activity of these two enzymes drops drastically (Foyer et al., 1998; Yanagisawa, 2014). NR is known to be regulated by light; however, the light intensity primarily regulates NR at the gene expression level (Lillo, 2008). The present observations suggest that LL intensity has a positive role in durum cultivars at low temperature, independently of the water supplies. The changes in Pn showed a light dependence similar to that of NR activity while they exhibited opposite responses to water deficiency. Both the present results and data in the literature (Nunes-Nesi et al., 2010) indicate that, when moderate water stress is combined with cold, their

simultaneous effect contributes to the maintenance of higher NR activities. However, the drought-induced increase in NR activity was only pronounced in the cold-tolerant Mv Makaróni plants. It seems that NL only has a positive effect on NR activity under non-stressed conditions, while reduced illumination helps to sustain nitrate reduction under unfavourable conditions.

A crucial step in the plant metabolism is the incorporation of reduced inorganic nitrogen forms into organic nitrogenous compounds. This primarily results in glutamine, which is catalysed by GS. Drought sensitivity and light dependence were previously reported in wheat (Nagy et al., 2013; Setién et al., 2013). In the two durum cultivars, the GS activity similarly decreased during water deprivation at low temperature. In contrast to NR, no differences were found in GS activity between well-irrigated and water-stressed plants at low temperature. In terms of light, GS activity was only higher in MvTD10-10 plants in the well-irrigated NL treatment at low temperature. A slight positive effect of NL on GS activity was also observed in cold-tolerant Mv Makaróni during mild water deprivation in the cold. The opposite was observed for Pn, which was significantly higher under LL than NL during water stress and cold conditions. LL may thus diminish the undesirable effects of water deficiency and suboptimal temperature in the case of Pn. Consequently, these results suggest that neither light intensity nor mild water deprivation have any facilitating effect on GS activity in these durum cultivars during cold stress.

The present data also suggest a possible connection between GS and invertase. In plants, sucrose and glucose may serve as signals for multiple metabolic pathways which are dependent on the nitrogen status and on environmental stimuli, such as light (Rosa et al., 2009). Since invertase was activated by light in MvTD10-10 plants in both irrigation regimes and GS also showed higher activity under NL in cold-sensitive MvTD10-10, the enhanced remobilisation of soluble sugar via sucrose degradation may support glutamine synthesis as either signal or substrate. The elevated level of soluble sugars produced by invertase may also contribute to sustaining the GS activity, as also found by Larios et al. (2004) and Oliveira and Coruzzi (1999). However, the effect of mild water deficiency could not compensate for the loss of GS activity at low temperature. Unlike GS, invertase was greatly enhanced by mild water deprivation at low temperature. These findings suggest that GS was influenced by other factors, such as the availability of energy sources (ATP) or a decreased rate of CO_2 fixation, rather than light. While Pn was lower under NL both in Mv Makaróni and MvTD10-10, invertase activity either did not decrease or was higher under NL at low temperature. These observations suggest that sugars remobilized from sucrose may compensate for the sugar deficit caused by the deceleration of sugar synthesis in the Calvin cycle under NL and cold.

Present results suggest that the differing cold tolerance of Mv Makaróni and MvTD10-10 can be explained by alterations in their RWC. The cold-sensitive genotype, MvTD10-10, had higher RWC, when exposed to the simultaneous effect of water deficiency and cold. The cold-tolerant cultivar Mv Makaróni was previously reported to be sensitive to drought (Bencze et al., 2013). This was confirmed by the lower RWC observed in this cultivar during water deficiency at low temperature. However, no changes in stomatal conductance were found between the cultivars, therefore this cannot explain the differences in RWC during water deprivation at low temperature. On the other hand, the enhanced activity of invertase induced by water deprivation in MvTD10-10 plants suggests a relationship to the altered water homeostasis in the cultivars, as demonstrated by the higher RWC in the leaves of MvTD10-10 in the cold. A rise in the soluble sugar content reduces the water potential, which could help to retain water in the cells (Rosa et al., 2009). On the other hand, decrease in RWC considered as a general response to cold, however, it manifests greatly in the later stage of cold-hardening or close to the freezing temperature (Ruelland et al., 2009). Other literature data report that monocot species lose water not pronouncedly at the early phase of cold-

hardening in contrast with drought. Freezing tolerant barley plants showed significant water loss under drought but not in the early stage of the cold-hardening (Faltusová-Kadlecová et al., 2002; Kadlecová-Faltusová and Faltus, 2001). Higher RWC content may expose MvTD10-10 to a lower level of cold-tolerance at suboptimal temperature. Thus, RWC level in the two durum cultivars could be regarded as an early response to cold which may alter after the long-term acclimation processes develop.

Besides water deprivation, NL also had a facilitating effect on invertase activity in MvTD10-10; however, the light-induced differences in this enzyme were not reflected in the RWC content. Opposite to the RWC, similar differences were found in the osmotic potential of the effect of light which suggests a light-dependent-regulation and a possible relationship between the invertase activity and osmotic potential. It was further confirmed, that water deprivation diminishes this light-dependent differences in osmotic potential in the cold. Characteristic differences of the effect of the light intensity and water supply, but not between the cultivars could be found in the measured parameters in the cold. It can thus be assumed that other defence mechanisms were induced in Mv Makaróni which are related to its better cold tolerance. In the case of nitrate assimilation, MvTD10-10 plants were found to be more effective than Mv Makaróni under well-watered conditions at both levels of illumination at 5°C. Similarly to the invertase activity, light-dependent difference in NR activity was diminished by the water deficit in the cold. These findings suggest that either MvTD10-10 reduces the deleterious effects of the early cold more effectively than Mv Makaróni, or Mv Makaróni keeps assimilation reduced as an early adaptive response to low temperature, until long-term adaptive responses develop. This denotes two different adaptive strategies. Comparisons between the cultivars could not elucidate the connection between the measured physiological parameters and the level of cold-tolerance.

Present findings demonstrate that 2-week-long cold-hardening affected the primer assimilation processes only but not the water homeostasis via RWC. Our findings are in accordance to Szűcs et al. (1999), i.e. adequate acclimation to cold requires longer hardening time in durum wheat than in bread wheat. Those physiological and biochemical processes which were affected by cold, such as Pn, YII, ETR and the activity of NR, could be improved by the moderate water deficit or LL. Besides, the existence of some priming effect caused by the water stress applied at 21°C might also play role in the better stress tolerance of the cultivars under cold conditions.

In conclusion, reduced light helps to overcome the undesirable effects of photoinhibition and sustain the rate of net photosynthesis and nitrate reduction at low temperature. The results also suggest a facilitating role of moderate water deficit at low temperature on nitrate assimilation and invertase activity, but not on the glutamine synthesis. Remobilisation of soluble sugars via sucrose degradation by invertase influenced the osmotic potential which might compensate for the cold- and drought-induced decline in photosynthetic sugar synthesis. Increase in the invertase activity as well in osmotic potential shows light-dependency which was diminished by the water deficiency at low temperature. Relation in the changes in RWC and osmotic potential was found only in the case of water loss while the effect of light and genotypes were dissimilar effect on these parameters. Present observations indicate that light intensity has an ambivalent role on plant life under adverse conditions. While normal light generally triggers stress defence responses, low light intensity rather contribute to the sustenance of assimilation under the simultaneous effect of water shortage and cold.

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Table 1 Relative water content (RWC) of the cold-tolerant Mv Makaróni and cold-sensitive MvTD10-10 durum cultivars at growth (21°C) and hardening (5°C) temperatures under normal irrigation (I), mild water deprivation (D), normal light (NL) and low light (LL) conditions. Data represent mean ± standard deviation of five plants per pot in each treatment. Different letters indicate statistically significant differences between the cultivars at $p \leq 0.05$, using Tukey's post hoc test.

Fig. 1 Changes in the net photosynthesis (Pn) (A) and stomatal conductance (gs) (B) of the cold-tolerant Mv Makaróni and cold-sensitive MvTD10-10 durum cultivars determined at a common laboratory temperature. Plants were grown at 21°C and hardening (5°C) temperatures under normal irrigation (I), mild water deprivation (D), normal light (NL) and low light (LL) conditions. Data represent mean ± standard deviation of five plants per pot in each treatment. Different letters indicate statistically significant differences between the cultivars at $p \leq 0.05$, using Tukey's post hoc test.

Fig. 2 Changes in the maximal quantum yield (Fv/Fm) (A), effective quantum yield of PSII [Y(II)] (B), electron transport rate (ETR) (C) and nonphotochemical quenching (NPQ) (D) of the cold-tolerant Mv Makaróni and cold-sensitive MvTD10-10 durum cultivars determined at a common laboratory temperature. Plants were grown at 21°C and hardening (5°C) temperatures under normal irrigation (I), mild water deprivation (D), normal light (NL) and low light (LL) conditions. On panel C, solid line indicates the mean of absorptivity and columns indicate ETR. Data represent mean ± standard deviation of five plants per pot in each treatment. Different letters indicate statistically significant differences in ETR between the cultivars at $p \leq 0.05$, using Tukey's post hoc test.

Fig. 3 Changes in the nitrate reductase (A) and glutamine synthetase (B) activity of the cold-tolerant Mv Makaróni and cold-sensitive MvTD10-10 durum cultivars at growth (21°C) and hardening (5°C) temperatures under normal irrigation (I), mild water deprivation (D), normal light (NL) and low light (LL) conditions. Data represent mean ± standard deviation of five plants per pot in each treatment. Different letters indicate statistically significant differences between the cultivars at $p \leq 0.05$, using Tukey's post hoc test.

Fig. 4 Changes in the invertase activity (A) and the osmotic potential (B) of the cold-tolerant Mv Makaróni and cold-sensitive MvTD10-10 durum cultivars at growth (21°C) and hardening (5°C) temperatures under normal irrigation (I), mild water deprivation (D), normal light (NL) and low light (LL) conditions. Data represent mean ± standard deviation of five plants per pot in each treatment. Different letters indicate statistically significant differences between the cultivars at $p \leq 0.05$, using Tukey's post hoc test.

Table 1

	21°C		5°C			
	I	D	I		D	
			NL	LL	NL	LL
Mv Makaróni	93.62 ± 1.57 [a]	43.54 ± 3.51 [c]	90.06 ± 7.30 [a]	84.55 ± 3.18 [a]	47.72 ± 4.85 [c]	51.23 ± 5.61 [c]
MvTD10-10	92.62 ± 4.48 [a]	45.50 ± 3.64 [c]	91.17 ± 1.99 [a]	91.09 ± 1.22 [a]	63.44 ± 4.08 [b]	63.10 ± 2.56 [b]