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# The effect of in vitro culture conditions on the pattern of maximum photochemical efficiency of photosystem II during acclimatisation of *Helleborus niger* plantlets to ex vitro conditions

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**Abstract** The influence of in vitro conditions including sucrose and nitrogen content in the culture medium as well as temperature on the pattern of chlorophyll *a* photochemical efficiency of photosystem II (PSII) of *Helleborus niger* L. plantlets upon transfer to ex vitro conditions were examined. In the experiment, the micro-shoots which were proliferated and rooted in vitro on the media with different levels of sucrose (10, 20, 30, 40, 50, 60, 70, and 80 g $L^{-1}$) and nitrogen salts $KNO_3$ and $NH_4NO_3$ (both 25, 50, 100 % according to MS medium), as well as temperature (15 and 20 °C) were used. The multiplication of axillary shoots were performed on modified MS medium supplemented with cytokinins (2iP, BAP and kinetin, each at a concentration of 1.0 mg $L^{-1}$) and $GA_3$ (2.5 mg $L^{-1}$) added together. For the rooting of shoots, the medium was supplemented with IBA 1 mg $L^{-1}$ and NAA 0.1 mg $L^{-1}$ added together. The maximal photochemical efficiency of PSII immediately upon removal of microplants from in vitro culture ranged from 0.24 to 0.81 depending on the treatment. High concentration of sucrose (above 50 g $L^{-1}$) and standard (100 %) or nitrogen salts decreased to 25 % in the MS medium as well as 20 °C temperature decrease the photochemical efficiency of microplants at transplanting time to ex vitro conditions as well as hinder acclimatisation and further growth out of culture. During acclimatisation to ex vitro conditions, a gradual increase of the maximal photochemical efficiency of in vitro-derived leaves was observed in all treatments. The maximal photochemical efficiency of PSII ranged from 0.79 to 0.83 after 4 weeks and from 0.81 to 0.83 after 20 weeks, indicating that *H. niger* plantlets gradually adapted to the greenhouse conditions and recovered their photosynthetic performance.

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**Keywords** Acclimatisation · Micropropagation · Nitrogen · Sucrose · Temperature

**Abbreviations**

| | |
|---|---|
| Fv/Fm | Variable fluorescence to maximum fluorescence ratio—maximum photochemical efficiency of photosystem II |
| PSII | Photosystem II |
| MS | Murashige and Skoog (1962) medium |

## Introduction

Although tissue culture methods create great opportunities in global horticulture, such as rapid production of high quality, disease-free, and uniform planting material, acclimatisation of microplants to greenhouse conditions still remains poorly understood and limits application of micropropagation technology for many plant species at a large scale (Hazarika 2006; Kumar and Rao 2012). In vitro conditions significantly differ from natural habitats of plants. Most in vitro cultures are characterised by low carbon dioxide inside vessels, low irradiance, high mineral and sugar contents in the culture medium, and high humidity, which affect plant morphology and physiology. Contrary to natural conditions, the plants cultured in vitro are subject to variable amounts of stresses: wounding, osmotic shock, hormonal imbalances, gas toxicities, sugar,

and nitrogen toxicity. The transition of microplants from in vitro to ex vitro conditions is the stage where plantlets suffer the greatest stress during the in vitro propagation process (Desjardins et al. 2009).

Chlorophyll *a* fluorescence is an indicator of photosynthetic reactions that take place in chloroplasts and is widely proposed as a useful indicator of plant quality during acclimatisation of microplants to ex vitro conditions (Carvalho et al. 2001; Serret et al. 2001; Borkowska 2006; Cassana et al. 2010; Osório et al. 2013; Sáez et al. 2013). The variable fluorescence to maximum fluorescence ratio (Fv/Fm) reflects the maximal efficiency of excitation energy capture by 'open' PSII reaction centres, and a decrease in this parameter indicates down-regulation of photosynthesis or photoinhibition. A significant decrease in the ratio of the Fv/Fm occurs at the time of micro-shoot removal from in vitro vessels; however, the scale of this decline can depend both on the composition of the in vitro medium and plant species. During acclimatisation, values of this parameter usually increase gradually, reflecting the gradual acclimatisation of microplants to new conditions.

Carbon and nitrogen levels in the culture medium as well as the carbon/nitrogen ratio significantly affect plant tissue growth (Caboche 1987; Vinterhalter et al. 2001; Ogura-Tsujita and Okubo 2006; Gabryszewska 2011, 2015) and affect many metabolic and developmental processes (Stitt et al. 2002; Badr et al. 2015). Some studies indicate also a potential role of carbon and nitrogen or their derived metabolites as signalling molecules (Coruzzi and Bush 2001; Rolland et al. 2002, 2006). Murashige and Skoog (1962) medium with 30 g $L^{-1}$ sucrose as a carbon source and $KNO_3$ (at 1900 mg $L^{-1}$) and $NH_4NO_3$ (at 1650 mg $L^{-1}$) is the most common medium for micropropagation of many horticultural plants. Sucrose is a source of carbon for plants in sterile, heterotrophic conditions inside the vessels and supports the maintenance of osmotic potential and conservation of water in cells. In turn, nitrogen plays a vital role in growth and differentiation of cultured tissues. There is some evidence that the level of sucrose in the culture medium combined with other in vitro factors can alleviate or intensify the potential photoinhibition of plantlets during acclimatisation (Serret and Trillas 2000; Fuentes et al. 2005a; Gago et al. 2014).

*Helleborus niger* L. (black hellebore) is a plant of high ornamental and medical value. It is an evergreen, winter-blooming perennial native to alpine areas and open woodlands in southern and central Europe. Effective micropropagation of *H. niger* regarding a comprehensive procedure of proliferation, rooting, and acclimatisation of microplants to greenhouse conditions leading to high quality and uniform plant material has not been yet attained (Lim and Kitto 1995; Seyring 2002; Aschan et al. 2005; Poupet et al. 2006; Dhooghe and Van Labeke 2007; Beruto et al. 2013). Therefore, an attempt was made to develop an effective method of *H. niger* micropropagation, and the first results of this study have been published (Gabryszewska 2015). In this study the influence of different levels of sucrose, nitrogen salts and temperature on multiplication and in vitro rooting of shoots was investigated. These results showed that the multiplication rate (from 1.8 to 3.7 axillary shoots per explant), the number of leaves (from 8.2 to 19.6 per multi-shoot), the leaf length (32–43 mm) and the fresh weight of multi-shoots (from 908 to 1863 mg) were strongly dependent on the sucrose and nitrogen salt relationship in the medium. The highest multiplication rate of axillary shoots (3.7) was found on the MS media supplemented with sucrose at a rate of 20–30 g $L^{-1}$ and nitrogen salt levels reduced to 50 %. In turn, increasing sucrose concentration to 50 g $L^{-1}$ strongly stimulated the number of roots per microplant (5.8–6.0) on the media with levels of nitrogen salts reduced to 25 or 50 %.

In this paper, the effects of in vitro culture conditions, such as sucrose and nitrogen salt concentration in the culture medium as well as temperature, on the pattern of photoinhibition during acclimatisation of *H. niger* to ex vitro conditions were described. Better understanding of the complex effects of the variables involved during the in vitro plant tissue growth on acclimatisation of microplants to greenhouse conditions should lead to a significant improvement of this process.

## Materials and methods

### Plant material and in vitro conditions

The present experiment is a continuation of earlier studies where the influence of various concentration of sucrose (10, 20, 30, 40, 50, 60, 70, 80 g $L^{-1}$), nitrogen salts: $KNO_3$ and $NH_4NO_3$ (both at 25, 50, and 100 % according to MS medium) as well as temperature (15 and 20 °C) on multiplication and in vitro rooting of shoots of *H. niger* were investigated (Gabryszewska 2015).

The initial explants and stabilization of axillary shoots culture were performed on modified Murashige and Skoog (1962) basal medium (50 % $KNO_3$ and 50 % $NH_4NO_3$ according to MS medium) containing various cytokinins (2iP, BAP, kinetin, each at concentration 1.0 mg $L^{-1}$) and $GA_3$ 2.5 mg $L^{-1}$ added together. The basal medium were supplemented with sucrose 20 g $L^{-1}$ and agar 2 g $L^{-1}$ + gelrite 1.2 g $L^{-1}$. The pH of the medium was adjusted to 5.8 before autoclaving. For proliferation, the culture of axillary shoots was subcultured on the fresh medium every 8–10 weeks. The experiment was conducted in vitro with shoots obtained from the sixth and seventh subcultures of

multiplication. In the last passage of multiplication stage (before the rooting experiment), the micro-shoots were proliferated (8 weeks) on the Murashige and Skoog (1962) medium with cytokinins (2iP, BAP, kinetin, each at concentration 1.0 mg $L^{-1}$) and $GA_3$ 2.5 mg $L^{-1}$ added together and supplemented with various concentration of sucrose and different levels of nitrogen salts and at temperature 15 or 20 °C. In the rooting in vitro experiment, single shoots, approximately 3–4 cm long, with 4.6–5.8 leaves were transferred to the Murashige and Skoog (1962) basal medium supplemented with IBA 1 mg $L^{-1}$ and NAA 0.1 mg $L^{-1}$ added together. After the rooting in vitro period (8 weeks), rooted plants has from 5.3 to 9.3 leaves and from 0.7 to 5.8 roots per shoot. The cultures were kept under light provided by cool-white fluorescent lamps (Philips TLD 36W/95) with a photosynthetic photon flux density (PPFD) of 80 µmol $m^{-2}$ $s^{-1}$. A 16-h photoperiod was maintained.

### Acclimatisation and ex vitro growth

Twenty uniform rooted micro-shoots for each of the three treatments (sucrose level × nitrogen salt level × temperature) were selected to evaluate the pattern of photoinhibition occurring during acclimatisation and the growth rate after 20 weeks of ex vitro cultivation (a total of 960 plantlets). Acclimatisation took place in the growth chamber during a period of 20 weeks. Plantlets from the root induction stage were transplanted to plastic boxes covered with transparent plastic caps to prevent dehydration. The substrate was a mixture of peat:perlite (3:1 v/v) at pH 6.5. The plastic covers were gradually opened to provide a relative humidity of 60 % at time T4 (4 weeks of ex vitro growth). Plant feeding began after 4 weeks using Symfo-vita (12-5-22-5 + micro, Pro-Lab) fertiliser. Every week, 100 mL of fertiliser solution per one box was applied. The concentration of fertiliser solution systematically increased, starting from 0.01 % after 4 weeks, 0.05 % after 8 weeks, and 0.1 % after 12 weeks of ex vitro cultivation. Plantlets were watered daily. Temperature in the growth chamber was kept constant (16–18 °C) throughout the light/darkness period, with a 16-h photoperiod. PPFD was 40 µmol $m^{-2}$ $s^{-1}$ during the first 2 weeks, and afterward it was progressively increased to 75 ± 10 µmol $m^{-2}$ $s^{-1}$. The experiment was carried out twice.

### Data acquisition

Chlorophyll *a* fluorescence was determined on attached leaves using a portable fluorimeter (PEA, Hansatech Industrustries, Ltd.). Measurements were taken on the in vitro-formed leaves three times during ex vitro growth [i.e., at T0 (immediately upon removal of plantlets from in vitro conditions), T4 (4 weeks after transplanting), and T20 (20 weeks after transplanting to ex vitro conditions)]. For each in vitro condition, chlorophyll *a* fluorescence emission from the upper leaf surface of 20 intact, dark-adapted leaves (30 min) was measured. Original ($F_0$) and maximum ($F_m$) fluorescence yields were measured to calculate variable fluorescence yield ($F_v$) by the equation of $F_v = F_m - F_0$. The Fv/Fm ratio was calculated as the maximum quantum yield of PSII photochemistry. At the end of ex vitro growth, after 20 weeks of plant cultivation, shoot and root fresh weights of plants were measured.

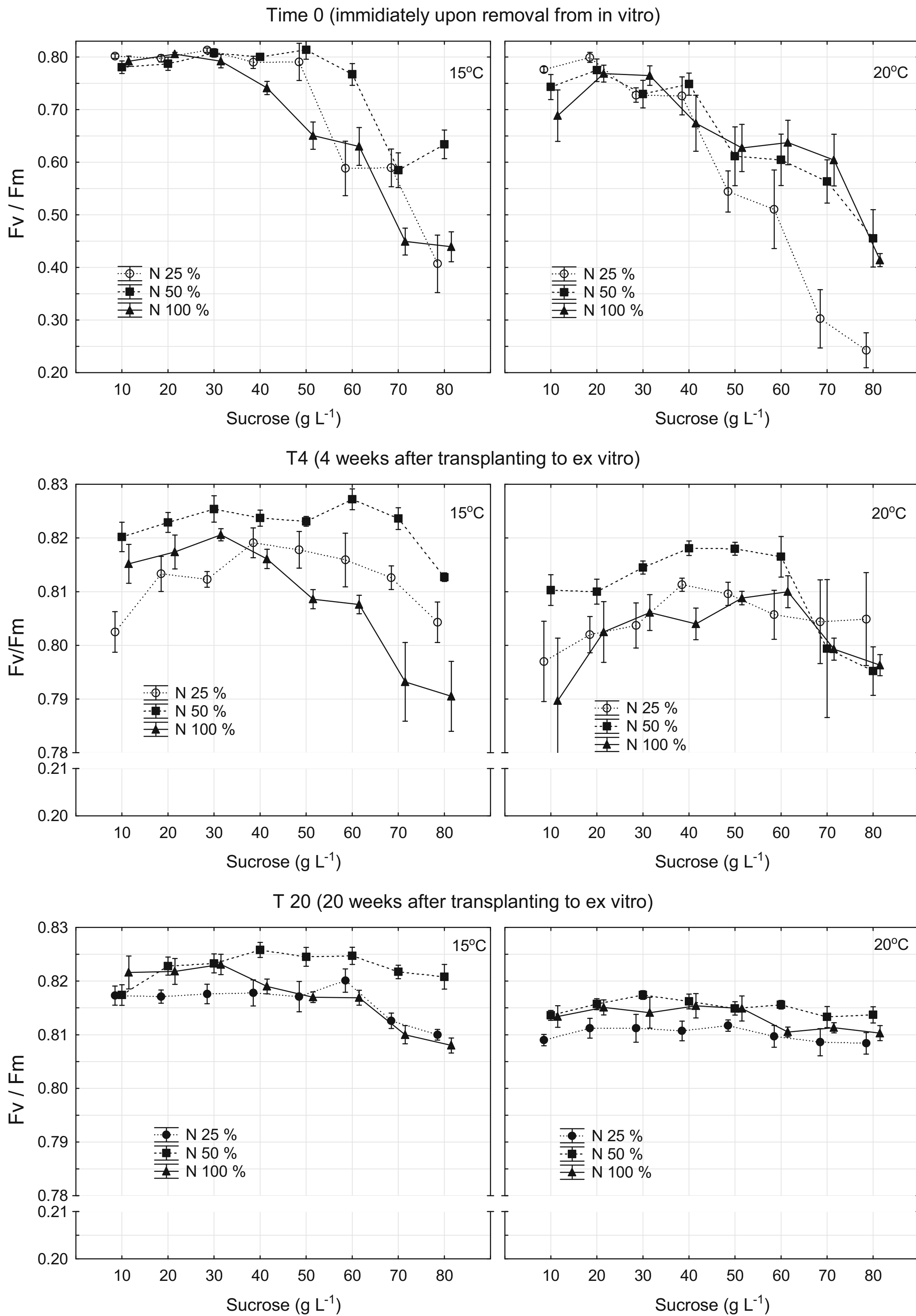
### Statistical analysis

The data from all the variables were subjected to three-way analysis of variance (ANOVA) with STATISTICA software. Tukey's honest significant difference (HSD) post-hoc test (at $p < 0.05$) for comparisons of means was used to investigate significant treatment effects.

## Results

All microplants of *H. niger* used in the study survived after transfer out of culture irrespective of the treatment; however, the in vitro condition in which they formed significantly affected their adaptation to ex vitro conditions and further growth.

Our study showed that the maximal photochemical efficiency (Fv/Fm) of microplants was strongly influenced by in vitro conditions. Overall, increasing sucrose concentration above 50 g $L^{-1}$, using standard (100 %) or very low nitrogen salts (25 %) in the rooting and proliferation media, as well as keeping high temperature in the phytotron (20 °C) significantly decreased the maximal photochemical efficiency of microplants at transplanting time to ex vitro conditions (T0), wherein interactions between the examined factors were also significant (Fig. 1). The maximum Fv/Fm for leaves immediately upon removal of microplants from in vitro culture ranged from 0.24 to 0.81. An Fv/Fm value in the range of 0.79–0.84 is the approximate optimal value for many plant species, with lowered values indicating plant stress (Maxwell and Johnson 2000). In our study, the Fv/Fm values >0.79 for in vitro plants were generally noted at lower temperature (15 °C) and sucrose concentrations in the range of 10–50 g $L^{-1}$ combined with decreased nitrogen salts to 50 or 25 % in the rooting and proliferation media (Fig. 1). Increasing sucrose concentration above 50 g $L^{-1}$ significantly decreased the maximal photochemical efficiency of microplants at transplanting time (T0), with the Fv/Fm value as low as 0.24 for plantlets grown on media supplemented with sucrose at the highest tested sucrose rate of 80 g $L^{-1}$,


Time 0 (immidiately upon removal from in vitro)
15ºC
20ºC
Fv / Fm
N 25 %
N 50 %
N 100 %
Sucrose (g L-1)
T4 (4 weeks after transplanting to ex vitro)
Fv/Fm
T 20 (20 weeks after transplanting to ex vitro)

◀**Fig. 1** The effect of sucrose (g $L^{-1}$), nitrogen salts (per cent of Murashige and Skoog medium), and temperature (°C) during in vitro shoot multiplication and root induction on the chlorophyll a fluorescence ratio Fv/Fm (variable to maximum fluorescence) measured on *Helleborus niger* leaflets during acclimatisation to ex vitro conditions at T0, immediately upon removal from in vitro conditions (*upper graph*), after 4 weeks (*middle graph*) and 20 weeks (*lower graph*) of ex vitro growth. Mean ± SE of 20 leaflets

nitrate salts reduced to 25 %, and high temperature (20 °C).

During acclimatisation to ex vitro conditions, gradual increasing of the maximal photochemical efficiency of in vitro-derived leaves was observed in all treatments. After 4 weeks (T4), the Fv/Fm values ranged from 0.79 to 0.83, indicating that *H. niger* plantlets gradually adapted to the greenhouse conditions and recovered their photosynthetic performance (Fig. 1). However, at this time, conditions posed in vitro still influenced the maximal photochemical efficiency of plantlets, with the highest rates of Fv/Fm noted for all tested sucrose concentrations except the highest concentrations (70–80 g $L^{-1}$) combined with 100 % supply of nitrogen salts in the medium and low temperature (15 °C) in the phytotron. A low Fv/Fm was also noted for plantlets derived at higher temperature (20 °C) on the media that contained the lowest sucrose concentration (10 mg $L^{-1}$) and the highest nitrogen salt supply (100 %). After 20 weeks of ex vitro growth (T20), the maximal photochemical efficiency of plantlets continued to increase for all treatments, although to a small extent, reaching values ranging from 0.81 to 0.83. At this stage of ex vitro growth, plants still affected the culture conditions in vitro. The highest values of Fv/Fm at this time, similar to those detected after 4 weeks of ex vitro growth, occurred for plants derived from in vitro culture that contained sucrose in the range of 40–60 g $L^{-1}$, decreased nitrogen salt concentration in the medium to 50 %, and temperature at 15 °C.

The physiological status of *H. niger* plants measured in our study by Fv/Fm generally corresponded to the rate of the weight gain of plants during establishment to ex vitro conditions. Plants showing the highest maximal photochemical efficiency during the whole 20-week period usually had the greatest plant fresh weight (Figs. 2, 3). After 20 weeks of growth in uniform ex vitro conditions, plants that had been micropropagated on media supplemented with sucrose at 20–50 g $L^{-1}$ with simultaneous reduction of nitrogen salts to 50 % and at a temperature maintained at 15 °C had the highest fresh weights of shoots (Fig. 2.). The effect of sucrose was more evident for root than for shoot growth, with optimal concentrations in the range of 30–50 g $L^{-1}$ (Fig. 3). Decreasing to 50 % nitrogen salt content in the medium for proliferation and rooting in vitro stimulated the growth of roots out of in vitro culture. Further reduction of nitrogen salts to 25 % was not conducive to the development of roots as it was for shoots. In contrast, regarding the growth of aboveground parts of plants, root growth was slightly but not significantly dependent on temperature set in vitro.

## Discussion

Until now, several studies have been published indicating poor photosynthetic ability of in vitro plants, which causes low survival and slow grow rates of plants after transfer from culture (Grout 1988; Matysiak and Nowak 1998; Dubé and Vidaver 1992; Hdider and Desjardins 1994; Van Huylenbroeck and Debergh 1996; Carvalho et al. 2001; Gaspar et al. 2002; Fuentes et al. 2005b; Hazarika 2006). A decline in Fv/Fm of PSII caused by down-regulation of photosynthesis or photoinhibition during the early days after transfer was reported in studies with micropropagated *Spathiphyllum floribundum* and *Calathea louisae* (Van Huylenbroeck et al. 1998), *Vitis vinifera* and *Castanea* spp. (Carvalho et al. 2001; Sáez et al. 2015), *Doritaenopsis* spp.(Jeon et al. 2006), *Alocasia amazonica* (Jo et al. 2009), *Morus nigra* (Ďurkovič et al. 2009a), and *Sorbus domestica* (Ďurkovič et al. 2009b). One of the factors that considerably diminishes the photosynthetic ability of plantlets cultured in vitro that resulted from a lower sink demand of these plants is high sucrose content (above 30 g $L^{-1}$) in the culture (Caboche 1987; Capellades et al. 1991; Serret et al. 1997; Fuentes et al. 2005b; Desjardins et al. 2009; Jo et al. 2009). Exogenous sugar in the plant system could suppress photosynthetic gene expression, reduce chlorophyll content, and reduce Calvin cycle enzymes as well as reduce Rubisco activity and Rubisco concentration, leading to low photosynthetic rates (Grout 1988; Hdider and Desjardins 1994; Van Huylenbroeck and Debergh 1996, Fuentes et al. 2005b) and induction of osmotic stress (Jo et al. 2009).

On the other hand, some authors believe that photosynthetic capacity of in vitro-cultured plants is sufficiently high to support autotrophic growth during periods of acclimatisation (Yue et al. 1992; Cassana et al. 2010; Eckstein et al. 2012). In some cases, exogenous sugars improve photosynthesis in vitro and facilitate acclimatisation to ex vitro conditions (Capellades et al. 1991; Van Huylenbroeck and Debergh 1996; Tichá et al. 1998, Pospíšilová et al. 1999; Badr et al. 2015). The effects of sugar, however, can be concentration-dependent. In *Arabidopsis thaliana*, exogenous sugars present in the medium at 3 % showed a positive effect on photosynthesis (Eckstein et al. 2012). At this concentration, they acted as photoprotectants, overcoming the negative influence of strong light on photosynthesis and the xanthophyll cycle. In tobacco, 3 %

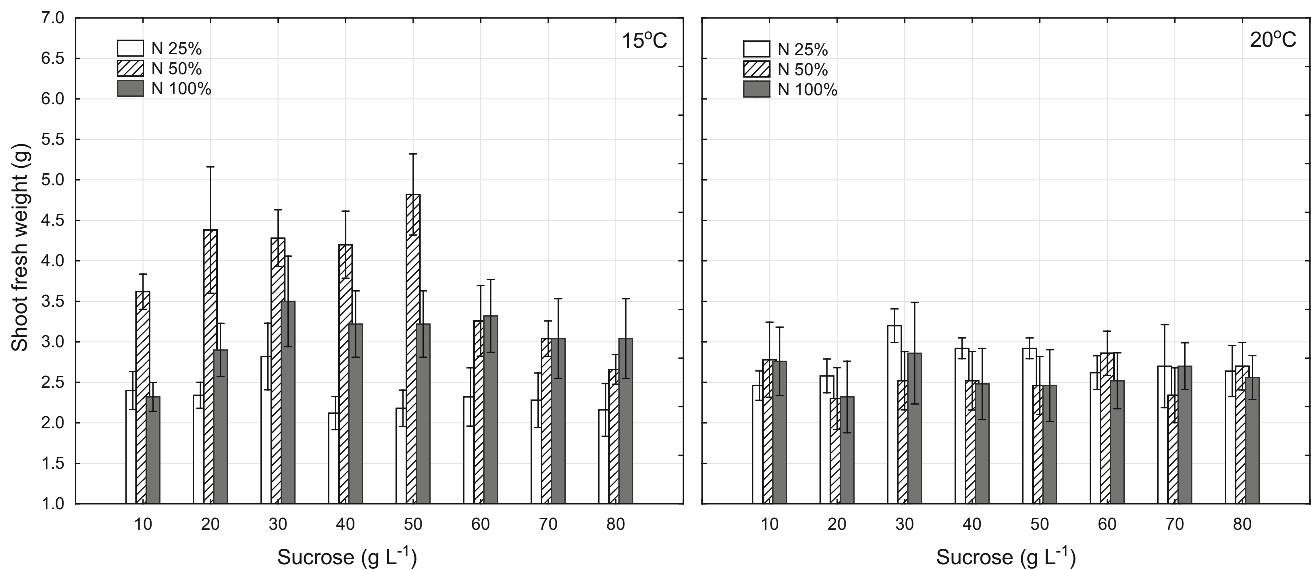



**Fig. 2** The effect of sucrose (g $L^{-1}$), nitrogen salts (per cent Murashige and Skoog medium), and temperature (°C) during in vitro shoot multiplication and root induction on the fresh weight of shoots of plants after 20 weeks of ex vitro growth. Mean ± SE of 20 leaflets

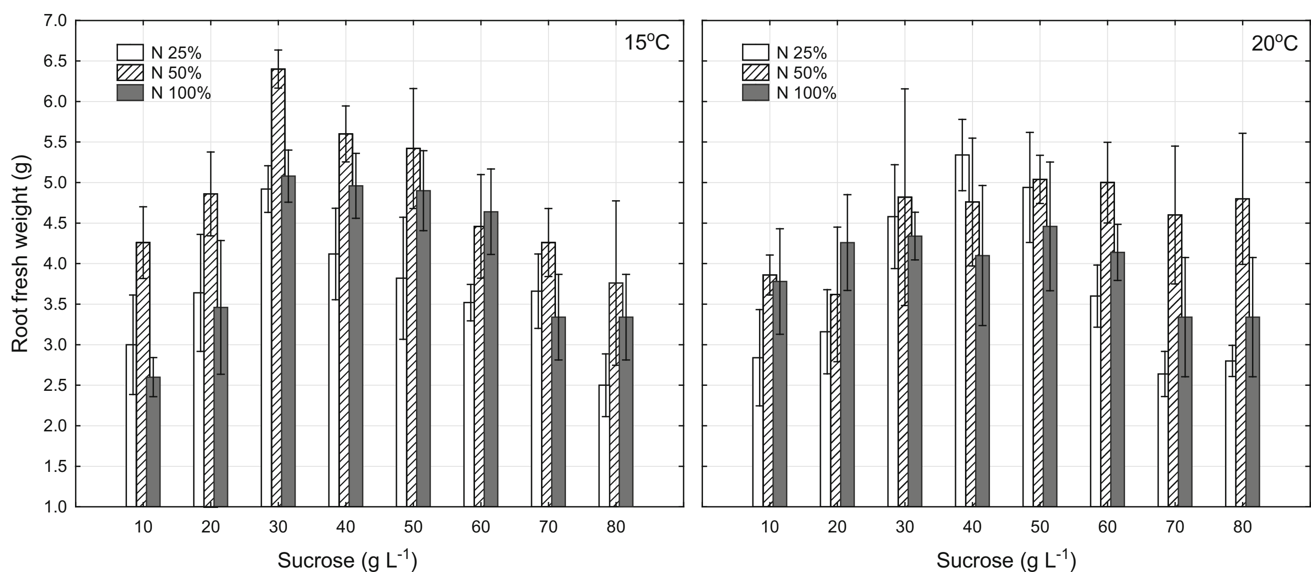



**Fig. 3** The effect of sucrose (g $L^{-1}$), nitrogen salts (per cent Murashige and Skoog medium), and temperature (°C) during in vitro shoot multiplication and root induction on the fresh weight of roots of plants after 20 weeks of ex vitro growth. Mean ± SE of 20 leaflets

sucrose enhanced photosynthetic activity and prevented photoinhibition, but a 5 % concentration caused a decrease in photosynthesis (Tichá et al. 1998). Many recent reports also indicated that photoautotrophic conditions (sugar-free medium, aerated containers) can be successfully used for in vitro propagation of many plant species (Xiao et al. 2011; Shin et al. 2013; Saldanha et al. 2014; Martins et al. 2015). Plants of *Bilbergia zebrina* cultivated in photoautotrophic conditions presented better growth and development, without anatomical and physiological disorders, compared to plants growing in the presence of sucrose (Martins et al. 2015).

It is well documented that nitrogen nutrition affects photosynthetic activity. Both deficiency and excess nitrogen usually down-regulate this process; however, this has not been exactly investigated with respect to in vitro-grown plants. In our study, microplants of *H. niger* at decreased-to-50 % nitrogen salts in MS medium showed the highest Fv/Fm of PSII, suggesting the highest photosynthetic performance of plantlets just after removal from culture. High

photosynthetic performance of these plants was maintained during 20 weeks of ex vitro growth, and plants at this time gained the highest weights of shoots and roots. Further reduction of nitrogen salts to 25 % in the MS medium decreased the Fv/Fm of PSII, which could be due to nitrogen deficiency and lower density of reaction centres (Lu et al. 2001; Li et al. 2012). Standard nitrogen salt levels in MS medium (100 %) proved to be adverse for in vitro growth of *H. niger* (Gabryszewska 2015), and it down-regulated photosynthetic performance of ex vitro micro-plants, as we have shown in this paper. Moreover, after 20 weeks of ex vitro growth, plants derived from 25 and 100 % nitrogen salts in MS media had lower shoot and root weight averages by 21 and 13 %, respectively, compared to those of MS media with 50 % reduced nitrogen salts. Another important issue is the relationship between nitrogen and sucrose in the in vitro culture medium. There is a close link between metabolism of carbon and nitrogen, which aims to maintain the constant balance between these elements in the plants. In our study, sucrose concentrations of 3–5 % combined with decreased nitrogen salts to 50 % in MS medium as well as temperature at 15 °C appeared to be optimal for maximum Fv/Fm of PSII for in vitro-grown *H. niger* plantlets.

Chlorophyll *a* fluorescence, an indication of the fate of excitation energy in the photosynthetic apparatus, has been often used as a reliable method for monitoring temperature stress and for screening plants for heat or chilling tolerance, including in in vitro conditions (Maxwell and Johnson 2000; Willits and Peet 2001). One of the first responses of a plant to environmental stress is an increase in nonradiative energy dissipation, which is reflected by the amount of chlorophyll *a* fluorescence. In our study, the highest Fv/Fm of PSII of *H. niger* plants at 20 °C was significantly lower than at 15 °C, demonstrating that this species is susceptible to high temperature in vitro. We suggest permanent damage to the PSII reaction centre or reduced de novo synthesis of the D1 protein under higher temperature (20 °C) rather than temporary inhibition, due to incomplete recovery of Fv/Fm during acclimatisation and further growth of plants out of culture.

## Conclusions

Overall, knowledge about the simultaneous impact of key factors during in vitro culture, such as sucrose, nitrogen concentration, and temperature, allows a more successful micropropagation system, reflected in better acclimatisation and *H. niger* plant quality. Thus, a sucrose concentration of 30–50 g $L^{-1}$ combined with decreased nitrogen salts to 50 % in MS medium and temperature at 15 °C appeared to be optimal for both in vitro growth and ex vitro acclimatisation of *H. niger* plantlets. High-quality micro-plants derived from such conditions more easily adapt to ex vitro conditions and exhibit faster growth in the natural environment. Finally, based on our results, we conclude that Fv/Fm can be an indicator of microplant development and photosynthetic performance during acclimatisation, which generally correspond with the rate of ex vitro growth.

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**Authors contribution statement** Bożena Matysiak: analysis and interpretation of data for the work, drafting the work, final approval of the version to be published, agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Eleonora Gabryszewska: developed the concept and designed experiments, performed the experiments with rooting in vitro of *Helleborus niger*, participated in literature search, helped in data interpretation and manuscript evaluation.

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