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Effect of different sucrose and nitrogen salt levels in the medium and temperature on in vitro propagation of *Helleborus niger* L.

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Abstract

Helleborus niger L. is a rhizomatous, herbaceous perennial with overwintering, divided, basal leaves. The objective of the study was to investigate the influence of different levels of sucrose (10, 20, 30, 40, 50, 60, 70, and 80 g l^{-1}) and nitrogen salts (25%, 50%, and 100% according to MS medium) as well as temperature (15°C, 20°C) on in vitro multiplication and rooting and ex vitro acclimatization of *H. niger*. The growth and multiplication of axillary shoots were performed on modified MS medium supplemented with various growth regulators (2iP, BAP and kinetin – each at a concentration of 1.0 mg l^{-1}, GA_3 2.5 mg l^{-1}). For the induction of roots, the medium was supplemented with IBA 1 mg l^{-1} and NAA 0.1 mg l^{-1}. Rooted plants were transplanted in a peat–perlite substrate (4:1) in a heated greenhouse for ex vitro acclimatization. The multiplication rate of *H. niger* shoots, in vitro rooting, and ex vitro acclimatization were strongly dependent on the sucrose/nitrogen salt relationship in the medium. The highest multiplication rate of axillary shoots (3.7) was found at a temperature of 15°C or 20°C, on the medium with cytokinins and GA_3 supplemented with sucrose 20–30 g l^{-1} and nitrogen salts at 50%. Sucrose at a concentration of 50 g l^{-1} strongly stimulated the number of roots per microplant (5.8–6.0) on the media with a reduced level of nitrogen salts (25% and 50%) when the temperatures were 20°C and 15°C, respectively. The plants rooted on the media with a high sucrose/nitrogen salt ratio showed acclimatization rates which ranged from 82% to 100%. Morphological observation of plantlets revealed obvious differences in leaf shape and size and the architecture of the root system as well as differences in the developmental stages of shoots grown on media with different sucrose and nitrogen salt concentrations.

Keywords: micropropagation; axillary shoots; *Helleborus niger*; nitrogen salts; sucrose; growing temperature; rooting

Introduction

The *Helleborus niger* L. belongs to the family Ranunculaceae [1–3]. The genus *Helleborus* comprises about 22 species which are distributed over different parts of Europe and West Asia. Only *H. thibetanus* is native to East Asia. *Helleborus niger* is classified in the section *Helleborus* [4–6]. Two morphological groups have been distinguished in the genus *Helleborus* according to caulogenesis: caulescentes and acaules [7]. The caulescent hellebores, including *H. lividus*, *H. foetidus*, and *H. argutifolius*, have above-ground stems supporting the leaves and flowers. The rhizome of these species is less developed. It is the opposite with the acaulescent group of *Helleborus* species. They are characterized by underground rhizomes that grow shoots with basal leaves and leafless flower stems with leaf-like bracts. *Helleborus niger* represents an intermediary between the caulescent and acaulescent hellebores [8].

Helleborus niger (Christmas rose) is a rhizomatous, herbaceous perennial with overwintering, divided, basal leaves. It is a species with an exceptionally long flowering period (2–6 months starting around Dec 25; Christmas) and relatively large flowers (6–13 cm), which change from white to green after fertilization [9]. This species is widely distributed in Southern and Central Europe [10]. *H. niger* is a variable taxon, but only two subspecies are generally recognized: *H. niger* ssp. *niger* and *H. niger* ssp. *macranthus* [4].

Christmas rose is important in commercial horticultural production as a garden perennial, blooming in winter and early spring. It is also important as pot and cut flowers. It is one of the most popular Christmas cut flowers cultivated in southern France and exported from France to Germany, the Netherlands, Switzerland, and Belgium [11]. Also, the other species are popular as garden perennials: *H. orientalis*, *H. viridis*, *H. lividus*, *H. foetidus*, *H. purpurascens*, and *H. argutifolius*. The flowers of the wild species are large and attractive, and they come in various shades of purple, green, and white [4]. Recently, some hybrid cultivars (*H. hybridus*) between different *Helleborus* species have been created. *Helleborus* species are also used as medicinal plants [12].

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Hellebores are propagated by seed and division. Generative propagation is limited because the seeds require several months to germinate after dispersing from the parent plants. The seeds of *H. niger* have deep, simple, morpho-physiological dormancy caused by the combination of rudimentary embryos and physiological dormancy that can be broken by cycles of warming and chilling [13]. Therefore, generative propagation requires special conditions and it takes time to create plants with a high degree of variation. Vegetative propagation is necessary to maintain the desirable characteristics of a particular hellebore cultivar. However, it has been reported that propagation through the division of rhizomes has a low multiplication rate and is time-consuming. The production of about 1000 plants from one single mother plant is possible within a period of 10–12 years [14]. In vitro propagation of many plants has played a very important role in rapid multiplication of cultivars and the production of healthy plants. Relatively little tissue culture research has been published on the micropropagation of hellebores [11,15–20]. The shoot multiplication efficacy of hellebores is influenced by several factors, such as: type of initial explants, genotype, growth regulators, and environmental factors [11,16,17].

Carbon (C) and nitrogen (N) are crucial for the growth and development of plants. Carbon metabolism is linked to nitrogen metabolism and the effect of a change in carbon abundance has an impact on nitrogen metabolism and vice versa. It has been suggested that C and N metabolism is modulated by the interaction of C signaling with N signaling or by C/N ratio signaling. Many developmental processes respond to carbon and nitrogen provisions: germination, leaf growth, flowering, root system architecture, and seed development. An integration of C/N nutrient signals with the phytohormone act to control C and N utilization. Among many plant growth regulators, the involvement of cytokinin in C/N regulation of plant growth and development has been demonstrated [21–27].

Some studies have presented the effects of carbohydrate and/or nitrogen on morphogenesis, growth, development, and photosynthetic activity of the following plants propagated in vitro: *Rosa*, *Cymbidium*, *Clematis pitcheri*, *Syringa vulgaris* and *Paeonia lactiflora* [28–38]. It has been recently found that tissue culture plantlets are constantly intoxicated by high concentrations of sucrose and nitrogen in the medium [38]. These high concentrations of sucrose and nitrogen salts are supra-optimal and stressful for many species, especially woody and perennial plants. For instance, a high concentration of sucrose in the medium has been determined to be detrimental for the photosynthetic activity in strawberry and other plants propagated in vitro [29,39].

The objective of the study was to investigate the influence of various levels of sucrose, nitrogen salts, and increasing temperature on morphogenesis and growth of *H. niger* during in vitro multiplication and rooting of shoots and in ex vitro acclimatization of microplants.

Material and methods

Plant material and initial culture

The mother plants of *Helleborus niger* L. were cultivated in a greenhouse. As explants, the axillary buds were isolated from the adult plants (2–3-year-old plants) and sterilized by soaking in commercial bleach (Ace 4 ml/water 100 ml) for 20 minutes. The initial explants and the subsequent subcultures of axillary shoots were performed on Murashige and Skoog [40] basal medium containing 2iP, BAP and kinetin (each at concentration 1.0 mg l^{-1}), GA_3 2.5 mg l^{-1}, sucrose 20 g l^{-1}, and agar 2 g l^{-1} + gerlite 1.2 g l^{-1}. The pH of the medium was adjusted to 5.8 before autoclaving. The axillary buds were cultured for an eight-week period and then transplanted to fresh medium. The culture of axillary shoots was subcultured on the fresh medium every 8–10 weeks.

Sucrose, nitrogen salts and temperature treatments

The study investigated the influence of sucrose (10, 20, 30, 40, 50, 60, 70, 80 g l^{-1}), nitrogen salts – KNO_3, NH_4NO_3 (25%, 50%, 100% according to MS medium) as well as temperature (15°C, 20°C) on in vitro multiplication and rooting and ex vitro acclimatization.

Shoot multiplication and rooting

During the experiments, axillary shoot multiplication was stimulated by various combinations of cytokinin (2iP, BAP and kinetin – each at a concentration of 1.0 mg l^{-1}) and GA_3 2.5 mg l^{-1} added to Murashige and Skoog [40] basal medium. For the induction of the roots on single microshoots, the Murashige and Skoog [40] basal medium was supplemented with IBA 1 mg l^{-1} and NAA 0.1 mg l^{-1}. The culture conditions were a 16 h photoperiod provided by cool-white fluorescent lamps (Philips TLD 36W/95) at 80 µmol m^{-2} s^{-1} and a temperature of 15°C or 20°C.

Acclimatization of microplants

Rooted plants from each treatment were transplanted in a peat–perlite substrate (4:1) under heated greenhouse conditions. These plantlets were protected by a piece of foil over a four-week period. The plants were checked to ensure that they were under constant humidity. Two weeks after the beginning of the acclimatization stage, the foil was gradually removed. Six months after acclimatization, the plantlets were ready to be transferred for cultivation.

Observations, measurements, and statistical analysis

Each treatment consisted of 3 jars with 5 explants. The experiment was repeated (the multiplication and rooting stage – 3 series). For the acclimatization stage, the experiment was repeated 2 times and from 18 to 42 rooted shoot were planted (the various number of rooted shoots in the combinations depended on the different rooting rate). The observations and measurements were recorded after 8 weeks of culturing or growth had taken place in the greenhouse. In the multiplication stage, the number of axillary shoots, the number of leaves and leaf length, and the fresh shoot weight were measured. After the rooting period (8 weeks), the number of roots, the number of leaves and leaf length were measured, and the fresh plantlets were weighed. During

acclimatization, the number of surviving plants was calculated. Morphological observation of *H. niger* plantlets was also carried out. The data were statistically analyzed and the means compared using Duncan's multiple range test.

Results

In this study, the effects of the sucrose concentration and nitrogen salts in the medium as well as the effect of temperature (15°C, 20°C) on in vitro multiplication and rooting of *H. niger* shoots, and ex vitro acclimatization of microplants were examined.

The axillary buds of *H. niger* cultured on the MS (1962) medium supplemented with BAP, 2iP, kinetin (each at a concentration of 1 mg l^{-1}), GA_3 2.5 mg l^{-1}, and sucrose 20 g l^{-1} developed shoots after about 2–3 months. Afterwards, the shoots were propagated by the development of axillary buds on the same medium that had been used for the initial explants.

Effect of sucrose, nitrogen salts and temperature on axillary shoot multiplication

The development of *H. niger* axillary shoots (multiplication rate) and their fresh weight were significantly influenced by the levels of sucrose and nitrogen salts in the media and by temperature. In the culture growing at a temperature of 15°C, the highest rate of axillary shoot multiplication (3.7) was found on the medium supplemented with sucrose 20 g l^{-1} and a half concentration of nitrogen salts (50% KNO_3 and 50% NH_4NO_3 according to the MS medium; Fig. 1a and Fig. 2). The same level of nitrogen salts but a

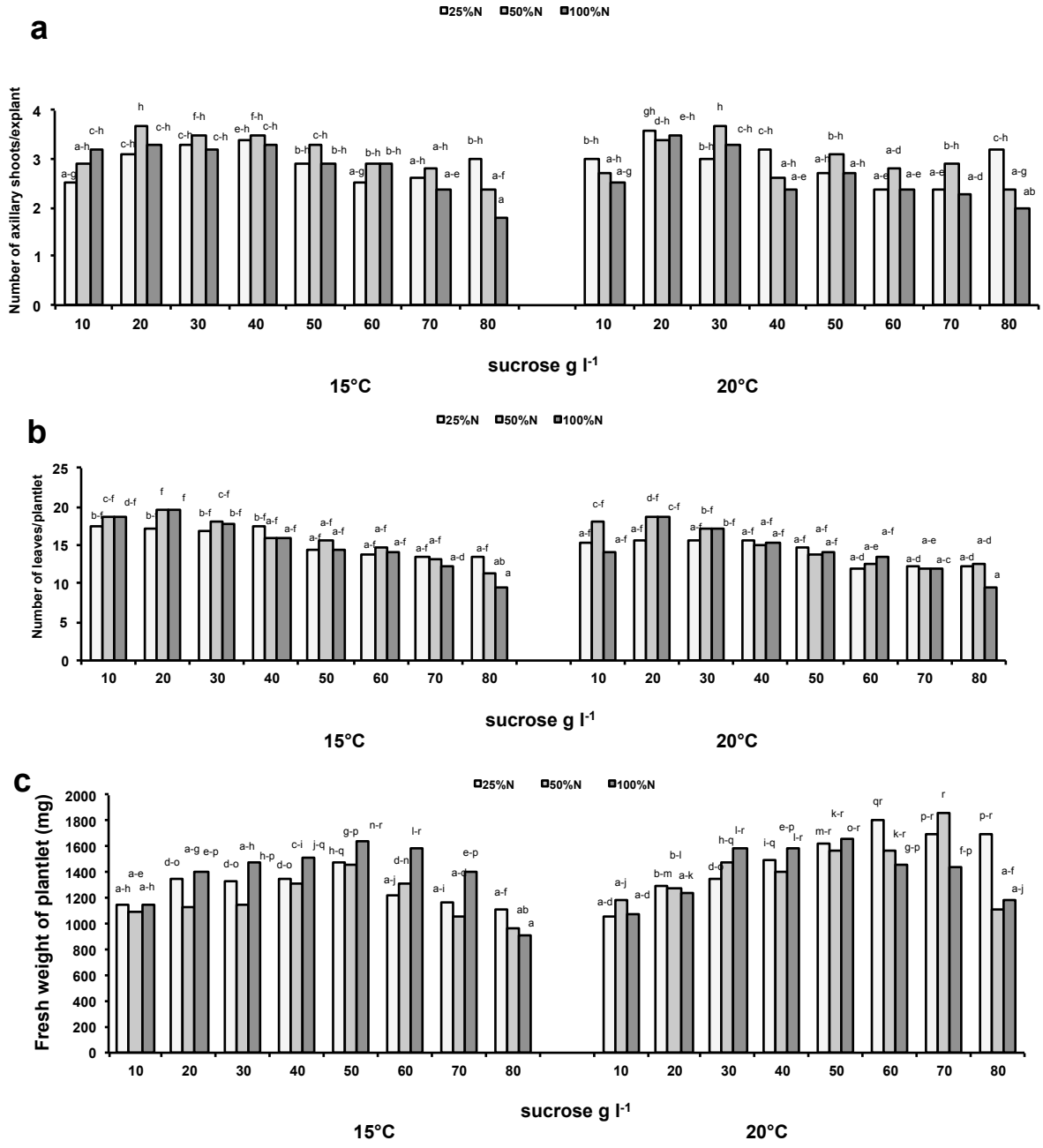

Fig. 1 The influence of sucrose, nitrogen salts, and temperature on the number of axillary shoots (**a**), the number of leaves in the plantlet (**b**), and the fresh weight of plantlet (**c**) of *H. niger* multiplicated in vitro.

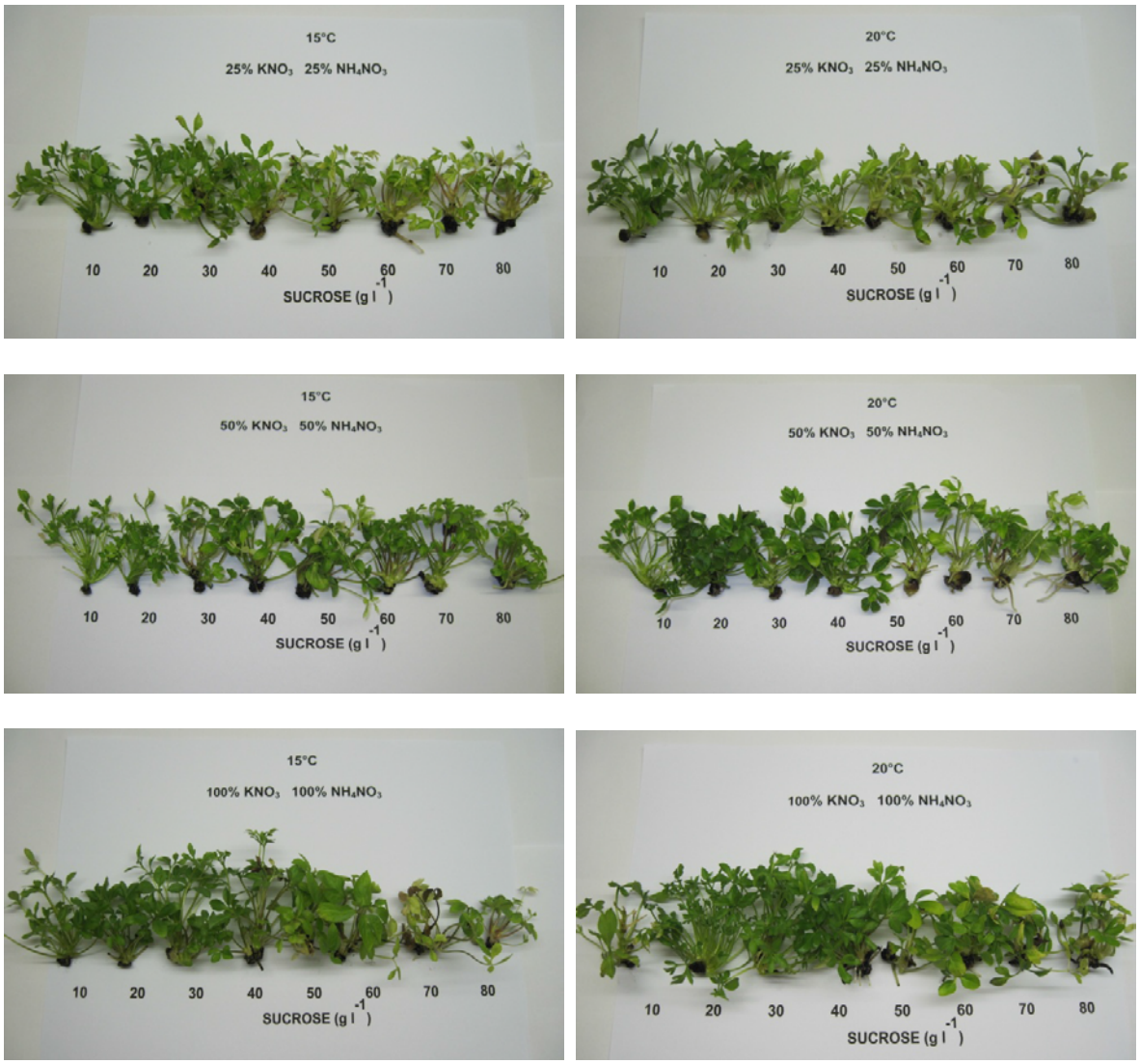

Fig. 2 The growth of *H. niger* shoots on MS media with different concentrations of sucrose and nitrogen salts (KNO_3, NH_4NO_3) at temperatures of 15°C and 20°C. The growth and multiplication of axillary shoots were performed on modified MS medium supplemented with various growth regulators (2iP, BAP and kinetin – each at a concentration of 1.0 mg l^{-1}, GA_3 2.5 mg l^{-1}).

higher content of sucrose (30 g l^{-1}) in the medium stimulated axillary shoot production (3.7) on the explants, when they grew at a temperature of 20°C. The sucrose concentration in the medium significantly increased the fresh weight of shoot cultures grown at 15°C and 20°C, but the interaction between the highest levels of sucrose and the nitrogen salts strongly inhibited the increase in fresh weight (Fig. 1c). Also, the sucrose/nitrogen salt ratio affected leaf development and growth in the hellebore plantlets. The plantlets grown in the media enriched with 20 g l^{-1} sucrose and supplemented with 50% or 100% of nitrogen salts showed the highest number of leaves (18.6–19.6; Fig. 1b, and Fig. 2). The enhanced supply of sucrose (from 60 to 80 g l^{-1}) in the media decreased the number of shoots and leaves on the plantlets, especially in the media enriched with a high level of nitrogen salts (Fig. 1a,b, and Fig. 2). The morphological observation of *H. niger* plantlets showed obvious differences in leaf shape and size as well as in the developmental stage of shoots growing on the media with different concentrations of sucrose and nitrogen salts (Fig. 3a–f, Fig. 4). An increased concentration of sucrose in the media affected the changes in developmental stage, such as the progression from the juvenile to the more adult phase, and the senescence of leaves (Fig. 3a–f). However, the effect of sugar was modified by the level of nitrogen salts in the media. In the media containing the highest supply of nitrogen salts (100%) and sucrose (70–80 g l^{-1}), the adult phase of shoots or senescence of leaves was observed. The ratio of sucrose/nitrogen salts also strongly influenced leaf area development (Fig. 4). A decrease was found in leaf area in response to a high level of sucrose (80 g l^{-1}) and a low supply of nitrogen salts (25%) in the medium. The concentration of sucrose and nitrogen salts in the media also affected the leaf shape. In the media with a low supply of sucrose (10–20 g l^{-1}) and a 25–50% supply of nitrogen salts, the leaf blades were divided into five segments. In contrast, an increase in the levels of sucrose and nitrogen salts in the media reduced the number of segments.

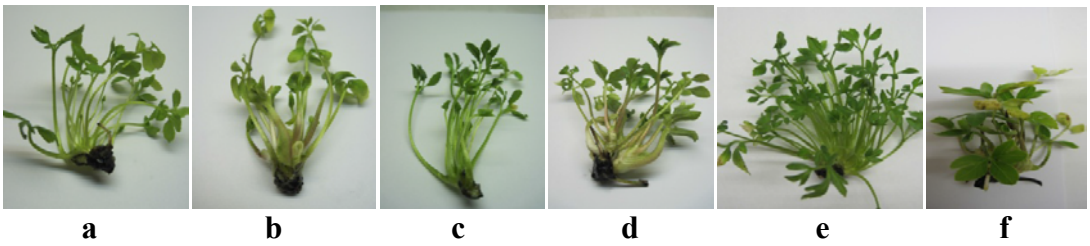

Fig. 3 Differences in the morphology of *H. niger* shoot growing at a temperature of 20°C and with various concentrations of sucrose and nitrogen salts in the media. KNO_3, NH_4NO_3 – 25% + sucrose 20 g l^{-1} (**a**); KNO_3, NH_4NO_3 – 25% + sucrose 80 g l^{-1} (**b**); KNO_3, NH_4NO_3 – 50% + sucrose 20 g l^{-1} (**c**), KNO_3, NH_4NO_3 – 50% + sucrose 80 g l^{-1} (**d**); KNO_3, NH_4NO_3 – 100% + sucrose 20 g l^{-1} (**e**); KNO_3, NH_4NO_3 – 100% + sucrose 80 g l^{-1} (**f**).

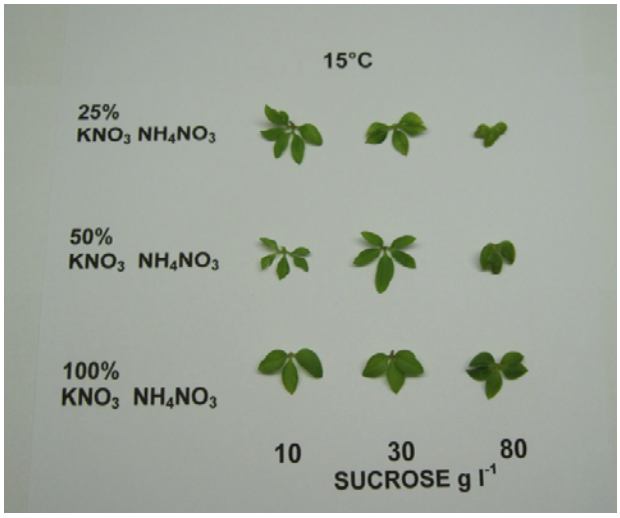

Fig. 4 Differences in the morphology of *H. niger* leaves depending on the concentration of sucrose and nitrogen salts in the medium.

Effect of sucrose, nitrogen salts and temperature on root formation

The rooting ability was strongly dependent on the sucrose/nitrogen salt relationship in the media. Sucrose at concentrations of 50 g l^{-1} strongly stimulated the number of roots per microplants (5.8–6.0) in the media with a reduced level of nitrogen salts (25% and 50%) when the microplants were kept at the temperatures of 20°C and 15°C, respectively (Fig. 5a). An increase in the level of nitrogen salts in the medium (100%) markedly inhibited root formation, especially at a temperature of 20°C. On the other hand, a high nitrogen content in the media promoted leaf formation, but an increase in sugar supply reduced the number of leaves (Fig. 5b). The best leaf growth on rooted shoots was found on the media containing a high ratio of nitrogen salts (100%) to sucrose (10–20 g l^{-1}). It is interesting to note that an increased supply of sucrose affected the architecture of the hellebore root system produced in vitro (Fig. 6). In the medium with a low concentration of sucrose, the roots grew horizontally. In contrast, high levels of sugar strongly affected the root morphology and influenced the number of roots. It was noted that the growth was more vertical, especially in the media with a higher supply of nitrogen salts.

The high sucrose levels (50 and 70 g l^{-1}) strongly increased leaf yellowing and senescence on the shoots in the multiplication phase as well as on the rooted microplants (Fig. 2, Fig. 3f, and Fig. 6).

Post-effect of sucrose, nitrogen salts and temperature on acclimatization of microplants

The well-rooted shoots were planted in peat–perlite substrate and transferred to a greenhouse for acclimatization (6–8 weeks). The percentage of plants that survived varied between 0% and 100% depending on the sucrose and nitrogen salts levels in the rooting media and depending on the temperature during the rooting phase (Fig. 7). The plants rooted in the media with the increased concentrations of sucrose and a low supply of nitrogen salts exhibited an acclimatization rate from 82% to 100% and there were more plants that survived after rooting when the temperature was 15°C. The survival rate of microplants rooted in the media with the highest content of nitrogen salts (100% according to MS medium) significantly varied (from 0% to 100%).

In the spring, after six months of growth in the greenhouse, the plants were transplanted to the field where they grew well (Fig. 8a). The first flowers were observed in the next year after planting *H. niger* in the field (Fig. 8b).

Discussion

Axillary bud development has proven to be the most often applied system for true-to-type in vitro propagation of plants. Shoot branching is the process by which axillary buds (dormant), located in the axil of a leaf, develop and form new branches (axillary shoots). Bud outgrowth is regulated by the interaction of environmental signals (temperature, light, sugar) and endogenous ones, such as plant hormones. In the present study, the axillary buds of *H. niger* developed shoots on MS medium supplemented with BAP, 2iP, kinetin, and GA_3. The axillary buds of four hellebore species (*H. argutifolius*, *H. foetidus*, *H. niger*, *H. orientalis*) were also used successfully for in vitro initiation on modified MS medium enriched with 2iP and BAP [17] or on MS medium supplemented with BAP, 2iP and NAA [19]. Poupet et al. [11] produced virus-indexed *H. niger* plants only from

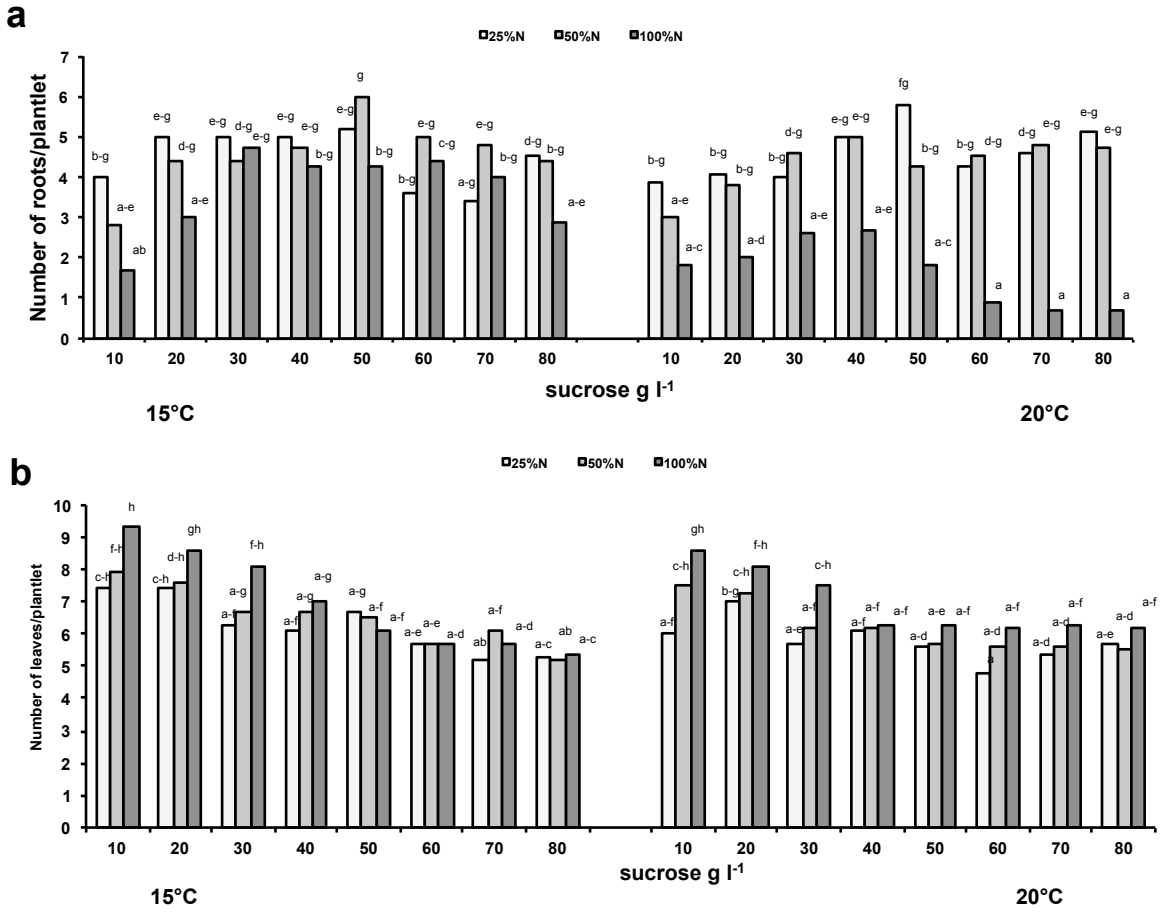

Fig. 5 The influence of sucrose, nitrogen salts, and temperature on the number of roots (**a**) and the number of leaves (**b**) on the shoot of *H. niger* rooted in vitro. For the induction of roots, the medium was supplemented with IBA 1 mg l^{-1} and NAA 0.1 mg l^{-1}. Explanation: see Fig. 1.

the apical meristem on medium containing: kinetin, GA_3, and IBA. Apical buds of *H. niger* seedlings were isolated and established on medium supplemented with BAP and GA_3 [16]. The genotype-dependence of the multiplication rates of some *Helleborus* species (*H. argutifolius*, *H. foetidus*, *H. niger*, *H. orientalis*) and selected clones of *H. niger* have already been observed [11,16,17]. The multiplication rate was significantly higher for *H. niger* than for *H. argutifolius*, *H. foetidus*, and *H. orientalis* [17].

The results presented in this paper demonstrate that axillary bud development of *H. niger* was affected by the sugar and nitrogen salt levels and their ratio in the media. Moderate levels of sucrose (20–30 g l^{-1}) and nitrogen salts (50% of KNO_3 and 50% of NH_4NO_3) in the media were beneficial for the development of axillary shoots and leaves. However, the plantlets growing in media with a lower or higher supply of sucrose and nitrogen salts showed different degrees with respect to their ability to cause the axillary shoots to proliferate. Only the highest level of sucrose (80 g l^{-1}) and nitrogen salts (100% of KNO_3 and 100% of NH_4NO_3) in the medium strongly and significantly inhibited axillary bud and leaf development as well as the increase in the fresh weight of the plantlets. Some studies have presented the effects of sucrose and/or nitrogen salts on axillary bud activity of in vitro propagated plants. In *Dracena fragrans* culture, axillary bud formation required high sucrose (50 g l^{-1}) levels in the medium. Furthermore, the increased levels of mineral salts in the media stimulated axillary branching. The synergistic effect of sucrose and inorganic salts in axillary bud formation was found [30]. In *Syringa vulgaris* propagated in vitro, the increased sucrose content in the media significantly reduced axillary bud development, but a high supply of nitrogen salts in the media counteracted the inhibitory effect of the sugar [37]. Similar results were obtained for the apical stem culture of *Clematis pitcheri* in which a low concentration of sucrose (10 g l^{-1}) and a 50% strength of nitrogen salts (according to MS medium) stimulated axillary branching; however, high levels of sucrose (30 g l^{-1}) inhibited the outgrowth of axillary buds [34]. Hence, the optimum sucrose/nitrogen salt ratio for axillary shoot branching depends on the species or genotype. It seems that the interaction of sugar, nitrogen and different phytohormones, especially gibberellin and cytokinins, plays an important role in the activation of axillary buds of hellebore.

The fresh weight of *H. niger* plantlets was stimulated by an increased level of sucrose, especially in the presence of a high level of nitrogen salts (100%) in the medium. *Helleborus niger* is a herbaceous perennial with overwintering leaves, and high sugar accumulation is probably involved in the cold acclimation process. In addition, an explanation

Fig. 6 The influence of different concentrations of sucrose and nitrogen salts (KNO_3, NH_4NO_3) on root formation in *H. niger* shoots growing in vitro at temperatures of 15°C and 20°C. For the induction of roots, the medium was supplemented with IBA 1 mg l^{-1} and NAA 0.1 mg l^{-1}.

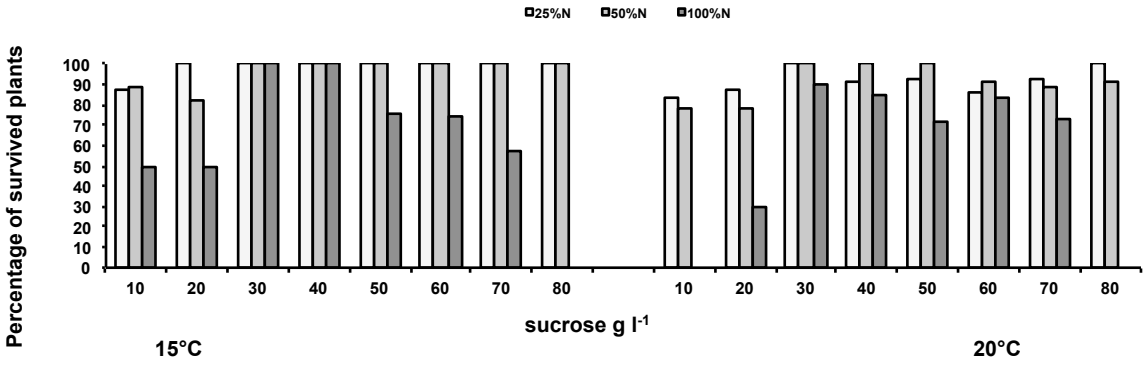

Fig. 7 The influence of sucrose, nitrogen salts, and temperature on the percentage of surviving plants of *H. niger* propagated in vitro. Explanation: see Fig. 1.

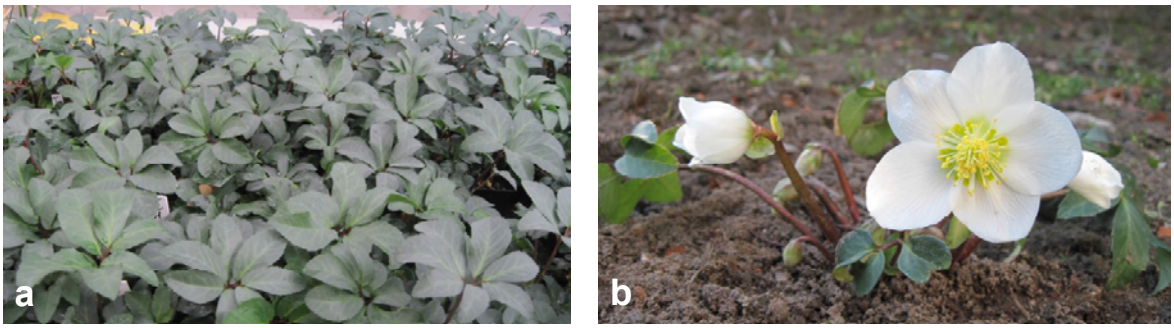

Fig. 8 Plants of *H. niger* propagated in vitro. **a** Six months after acclimatization in the greenhouse. **b** First flowers in the first year after acclimatization in the greenhouse.

for the high sucrose requirements of *H. niger* propagated in vitro might be the specific photosynthetic activity of this species in natural conditions. Although leaves are traditionally considered as the main sources of photosynthates, the reproductive structures of *Helleborus* are also photosynthetically active and therefore can fix substantial amounts of carbon. An additional amount of carbon supplied from floral photosynthesis has been observed for *H. viridis* and *H. niger* [9,41]. On the other hand, it has been found that tissue culture plantlets are constantly intoxicated by high concentrations of sucrose and nitrogen in the medium [38]. For many plant species propagated in vitro, a high concentration of sucrose (above 30 g l^{-1}) in the media was detrimental for the growth and photosynthetic activity [29,38,39]. It is known that sugar alone or through its interaction with different phytohormones (GA, cytokinins, ABA, ethylene) and nitrogen signal can induce or suppress many growth-related gene responses in higher plants [22, 24,42–45]. For instance, increased sugar levels delay seed germination and stimulate the induction of flowering and senescence in some plant species. Higher sugar concentrations can also increase the number of tubers formed by potatoes and can stimulate the formation of adventitious roots. Soluble sugars also effect the formation of more adult structures, such as leaves, nodules, pollen, tubers, and roots [44]. An increase in the sucrose concentration in the media stimulated lily bulb formation [46,47], corm induction in *Watsonia vanderspuyiae* [48], microtuber formation in *Xanthosoma sagittifolium* [49], microrhizome production in *Zingiber officinale* [50], and renewal buds formation in *Paeonia lactiflora* [36]. Sugars also help to regulate the timing of the developmental phase changes, such as the progression from juvenile to adult phases, flowering, and senescence. In lily bulblets regenerated in vitro, the influence of sucrose on maturation was evident from the fact that more stems were formed in bulblets regenerated when the sucrose level was high than when the sucrose level was low. The MS mineral salts stimulate bulb growth but do not seem to affect the phase change [51]. In contrast, the developmental phase of the *H. niger* in vitro shoot was strongly dependent on the ratio of sucrose/nitrogen salts. An increased concentration of sucrose in the media affected the development of hellebore shoots from the juvenile to the more adult phase. However, the effect of sugar is modified by the level of nitrogen salts in the media. It seems that the phase change may be regulated more by C/N ratios than by absolute sugar levels. Plants have evolved a sensory system that interacts to regulate C and N assimilation, metabolism, transport, and developmental events [23,25,26]. Increased concentration of sucrose in the medium (with cytokinins and GA_3) affected leaf yellowing and senescence in *H. niger*. The highest supply of sucrose (70–80 g l^{-1}) in the media accelerated leaf yellowing and senescence, while none of the nitrogen levels in the medium accelerated these symptoms. It is interesting to note that even a high nitrogen supply in the media did not prevent sucrose-induced leaf senescence. In *Arabidopsis* plants, an external supply of glucose in combination with a low nitrogen supply induced leaf yellowing and changes in gene expression that are characteristic of developmental senescence [52]. In the same plant species, the supply of nitrogen was found to be able to reverse senescence processes. For example, leaves of *Hordeum vulgare* and *Arabidopsis thaliana* showed a reversion of the senescence processes after receiving an additional supply of nitrogen [53]. These results suggest that senescence may be regulated more by C/N ratio than by absolute sugar or nitrogen levels.

The different levels of sucrose and nitrogen salts were observed to affect the size and shape of *H. niger* leaf in vitro. The decrease in leaf area was a response to the high supply of sucrose and the low level of nitrogen salts in the media. The negative effects of sucrose on leaf blade size can be reduced by the addition of higher levels of nitrogen salts to the media. In contrast, for *Ceratonia siliqua* grown in vitro it was sucrose which strongly stimulated leaf area development, but when the level of nitrogen salts in the media was decreased it caused a rapid reduction in leaf size [31]. These results suggest that leaf growth is partially dependent on carbon/nitrogen availability. The leaves on mature *H. niger* plants are divided into 5–10 leaflets [54], but during in vitro propagation the number of leaf segments was reduced to 3–5. This finding is probably associated with the early growth stage of hellebore plants which showed marked juvenile traits.

A few published reports on in vitro propagation of *Helleborus* note that rooting and acclimatization of microplants are not yet problem-free [19]. The rooting ability of various

Helleborus species has been investigated in vitro and ex vitro [11,15–18]. Studies on in vitro rooting have shown that cultivation of *H. niger* shoots on IBA and NAA-containing media resulted in a higher proportion of rooted shoots than when cultivation took place on auxin-free medium. The highest rooting rates (96.4%) and good root formation were recorded on IBA-containing media [16]. In contrast, various auxins (IAA, IBA, NAA) did not work well and explants of *H. niger* only formed root calli [11]. High root formation (more than 96%) was induced using various concentrations of IAA in two rooting phases [11]. For *H. niger*, a satisfactory survival rate for rooting in vitro (87%) and ex vitro (85%) was obtained after chilling (7°C) microshoots in an IBA and NAA-containing solution for 7 days [18]. A low survival percentage (50–56%) was found for ex vitro rooting of *H. orientalis* [15]. Dhooghe and van Labeke [17] suggested ex vitro rooting for four *Helleborus* species which were induced for one week by drenching them in a solution of IBA and NAA at 5°C, but no data on the rooting rate and survival percentage was provided.

Among growth regulators, auxin, cytokinin, and abscisic acid have been implicated most strongly in regulating lateral root formation, and in modulating lateral root formation in response to environmental cues [55]. The plant hormone auxin appears to play a critical role in lateral root initiation. Also, carbohydrates and nitrogen have been reported as having an influence on adventitious root initiation. It was found that in *Arabidopsis* culture the formation of lateral roots and their emergence are strongly influenced by the take-up of sucrose from the medium. These data also show that increased sucrose metabolism leads to a developmental switch to create a more highly branched root system architecture [55]. However, lateral root initiation in *Arabidipsis* was repressed by a high sucrose to nitrogen ratio in the growth media [56]. A low nitrate supply promotes lateral root growth in *Arabidipsis* but has no effect on primary root growth. On the other hand, a high supply of nitrate inhibited lateral root development when seedlings were grown on the media containing a low concentration of sucrose [57].

Relatively few studies have been conducted on the effect of the sucrose/nitrogen salt ratio in the media on the initiation and growth of roots in vitro [36,58,59,60,61]. The results presented in this paper demonstrate that root formation on *H. niger* microshoots was strongly dependent on the level of sucrose and nitrogen salt in media containing IBA 1 mg l^{-1} and NAA 0.1 mg l^{-1}. Increasing the sucrose concentration, from 10 to 50 g l^{-1}, strongly promoted rooting. The best root formation was found in media the containing sucrose at a concentration of 50 g l^{-1} and with a relatively low level of nitrogen salts (25–50% according to MS medium). The higher level of nitrogen salts (100% according to MS medium) in the media and shoot exposure to a low level of nitrogen (10–30 g l^{-1}) resulted in a strong suppression of root induction and root development, especially at a higher temperature (20°C). The ratio of sucrose to nitrogen also affected the architecture of the *H. niger* root system produced in vitro. An increased sugar level strongly influenced a more vertical growth of the roots, mainly in the media with a high supply of nitrogen salts. In contrast, in the media with a low concentration of sucrose the roots grew horizontally. In rose microshoots grown in vitro, a high sucrose to nitrogen ratio in the media favors root formation and the ionic form of nitrogen appears to have a significant effect on root initiation [60]. In sugar-beet hypocotyls grown in vitro, root formation was stimulated by increased levels of sucrose or inorganic nitrogen if IAA was present in a high concentration in the medium. However, no stimulation was caused when a low concentration of IAA was present in the medium [58]. The presence of low levels of nitrogen salts in the medium (12.5% according to MS medium) and an increased concentration of sucrose (from 10 to 30 g l^{-1}) promoted root formation on *Paeonia lactiflora* shoots, mainly in the presence of IBA and NAA in the medium [61]. Adventitious root production on *Pelargonium* petioles was inhibited by increasing concentrations of nitrogen and sucrose in the medium [59].

Therefore, it is possible that the ratio of sucrose to nitrogen is a key factor in regulating lateral root initiation in *Arabidopsis* and other plant species. The dual function of sugar and nitrogen, as a nutrient and a signaling molecule, significantly complicates analysis of the root formation mechanism.

Conclusions

The results presented here show that different levels of sucrose and nitrogen salts in MS media and their interaction with plant growth regulators play an important role in axillary shoot development, lateral root initiation in vitro and ex vitro acclimatization of *H. niger* plants.

Moderate levels of sucrose (20–30 g l^{-1}) and nitrogen salts (50% of KNO_3 and of 50% NH_4NO_3) in the media were beneficial for the development of axillary shoots and leaves. In contrast, high sucrose and nitrogen salt levels in the media resulted in the highest rate of rooting and acclimatization of microplants. Different sucrose/nitrogen salts ratios in the MS medium and different temperatures affected the morphology of the shoots and roots of *H. niger* plantlets propagated in vitro.

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Competing interests

No competing interests have been declared.

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Wpływ różnych stężeń sacharozy i soli azotu oraz temperatury na rozmnażanie in vitro *Helleborus niger* L.

Streszczenie

Gatunek *Helleborus niger* L. (ciemiernik biały) należy do rodziny Ranunculaceae (jaskrowate). Jest zimozieloną byliną posiadającą krótkie, grube, rozgałęzione kłącze. Wykorzystywany jest głównie jako bylina ogrodowa, ale coraz powszechniej uprawia się go na kwiaty cięte i jako rośliny doniczkowe. Celem badań było określenie wpływu różnych stężeń sacharozy (10, 20, 30, 40, 50, 60, 70 i 80 g l^{-1}) i soli azotu (25%, 50%, 100% wg składu pożywki MS) oraz temperatury (15°C, 20°C) na namnażanie i ukorzenianie pędów *H. niger* in vitro oraz aklimatyzację mikrosadzonek ex vitro. Wzrost i namnażanie pędów kątowych prowadzono na pożywce MS zawierającej cytokininy (2iP, BAP i kinetynę – każda w stężeniu 1.0 mg l^{-1}) i GA_3 2.5 mg l^{-1}. Do indukcji ukorzeniania stosowano pożywkę MS zawierającą IBA 1 mg l^{-1} i NAA 0.1 mg l^{-1}. Ukorzenione pędy ciemiernika purpurowego sadzono do podłoża zawierającego torf i perlit (4:1) i umieszczano w szklarni w celu aklimatyzacji. Współczynnik namnażania pędów, ich zdolność ukorzeniania in vitro i aklimatyzacja ex vitro istotnie zależały od wzajemnej relacji sacharozy/soli azotu w pożywce. Największą wartość współczynnika namnażania pędów kątowych (3.7) uzyskano w temperaturze 15°C lub 20°C, na pożywce z cytokininami i GA_3 zawierającej sacharozę 20–30 g l^{-1} i sole azotu w stężeniu 50%. Sacharoza w stężeniu 50 g l^{-1} silnie stymulowała powstawanie korzeni na pędach (5.8–6.0) rosnących na pożywce ze zredukowanym poziomem soli azotu (25% i 50%) zarówno w temperaturze 20°C i 15°C. Rośliny ukorzeniane na pożywkach zawierających wysoką relację sacharozy do soli azotu wykazywały dużą zdolność aklimatyzacji w szklarni (82–100%). Różna zawartość sacharozy/soli azotu w pożywce wpływała na kształt i wielkość liści, architekturę systemu korzeniowego i fazę rozwojową roślin rozmnażanych in vitro.