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PROPAGATION *in vitro* OF HELLEBORES (*Helleborus* L.) REVIEW

Eleonora Gabryszewska✉

Research Institute of Horticulture, Konstytucji 3 Maja 1/3, 96-100 Skierniewice, Poland

ABSTRACT

The genus *Helleborus* belongs to the family Ranunculaceae and comprises about 22 species, which are distributed over different parts of Europe and West Asia. In Poland, only *H. purpurascens* is native and it occurs in the Western Bieszczady Mountains. Hellebores are popular as ornamental cut flowers and medicinal plants in Europe and the USA. Conventional propagation by seeds or division has a low multiplication rate and is time-consuming. Vegetative propagation is necessary to maintain the desirable characteristic of a particular hellebore cultivar. Although some research on tissue culture of hellebores has been published, effective commercial micropropagation of these species has not been attained because cultivation *in vitro* is still very difficult. This review presents the progress in *Helleborus* species propagation *in vitro* for its commercial production. The efficacy of hellebore micropropagation (initiation and stabilization of culture, multiplication and rooting *in vitro* and acclimatization *ex vitro*) has been influenced by several factors, such as: type of initial explants, genotype, growth regulators, and environmental factors (temperature, sucrose, nitrogen salts, phosphorus). The genotype-dependence of multiplication and rooting *in vitro*, and acclimatization *ex vitro* of some *Helleborus* species has been presented.

Key words: *Helleborus*, micropropagation, axillary shoot branching, rooting, acclimatization *ex vitro*, growth regulators, environmental factors

Abbreviations: BAP – 6-benzylaminopurine, 2iP – 6-(γ,γ-dimethylallylamino)purine, TDZ – thidiazuron, IAA – indole-3-acetic acid, IBA – indole-3-butyric-acid, NOA – 2-naphthoxy acetic acid, NAA – α-naphthalene acetic acid, GA_3 – gibberellic acid, MS – Murashige and Skoog [1962] medium, WPM – Woody Plant Medium [Lloyd and McCown 1980], U-medium [Haensch 1999].

INTRODUCTION

Botanical background

The genus *Helleborus* belongs to the family Ranunculaceae and comprises about 22 species, which are distributed over different parts of Europe and West Asia. The majority of *Helleborus* species are concentrated in the Balkan region (fig. 1). Only *H. thibetanus* is native to East Asia [Tutin 1964, Nowicke and Skvarla 1983]. In Poland, only *H. purpurascens* is native and it occurs in the Western Bieszczady Mountains (Eastern Carpathians) [Ralska-Jasiewiczowa 1960, Szucki 1982, Mitka and Bochenek 1998, Bochenek 1998, Mitka et al. 2001, Mitka and Michalik 2008, Suder 2010]. *H. purpurascens* is a rare and endangered element of Polish flora, being protected by law [Mirek and Piękoś-Mirkowa 2008].

These perennials are all diploids (2n = 2x = 32) [Benett and Smith 1976, Zonneveld 2001, Meiners et

✉eleonora.gabryszewska@inhort.pl

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Fig. 1. Hellebores growing in its natural habitat in the mountains of south-western Serbia. Photo: E. Gabryszewska

al. 2011]. Mathew [1989] divided the genus *Helleborus* into six sections (Syncarpus, Griphopus, Chenopus, Helleborus, Helleborastrum, Dicarpon) according to plant structure, the ability to hybridise, pollen morphology and seed characteristics. By contrast, Werner and Ebel [1994] divided the genus *Helleborus* into two subgenera, *Helleborus* and *Helleborastrum,* according to the species' hypsophylls**.** The sections Griphopus, Chenopus and Helleborus belong to the subgenus *Helleborus*, but the sections Syncarpus, Dicarpon and Helleborastrum to the subgenus *Helleborastrum.* Analysis of the DNA contents in *Helleborus* species supported the previously suggested classification of the genus into six sections [Meiners et al. 2011]. *H. niger* is classified into the section Helleborus, but *H. purpurascens* and *H. orientalis* into the section Helleborastrum [Mathew 1989, Zonneveld 2001, Meiners et al. 2011]. Two morphological groups have been distinguished in the genus *Helleborus* according to caulogenesis: the caulescentes and acaules [Braun and Bouché 1861]. The caulescent hellebores, including *H. lividus*, *H. foetidus*, and *H. argutifolius*, have above-ground stems supporting the leaves and flowers. They have a large terminal inflorescence with many flowers. The rhizome of these species is less developed. The opposite is true for the acaulescent group of *Helleborus* species (for example: *H. purpurascens, H. orientalis, H. atrorubens, H. cyclophyllus, H. torquatus)*. They are characterised by underground rhizomes that grow shoots with basal leaves, and leafless flower stems with leaf-like bracts [McLewin and Mathew 1995]. *H. thibetanus* can be considered acaulescent by its basic appearance, but the rhizome and the roots are atypical for this group because the inflorescence appears before the true leaves start to expand [McLewin and Mathew 1995]. *H. niger* represents an intermediary between the caulescent and acaulescent hellebores [McLewin and Mathew 1995].

Hellebores in floriculture

Hellebores are popular as ornamental perennial pot plants and cut flowers in Europe and the USA. Hellebores are interesting for their early flowering, beginning as early as December in some species, and flowers, which last for 2 to 3 months and come in a multitude of colours, from whites, yellows, and greens to pinks, plums, purples, blue-blacks and even spotted forms. The flowers appear in single, semi-double, or double forms. Unlike in most plants, the petals of hellebores are not showy, but are modified into short nectaries. Hellebores are easy to cultivate in the garden and have enjoyed increasing popularity in Europe, the United States and other countries of the world. The two most popular *Helleborus* species in horticulture are the Christmas rose (*H. niger* Linn.) and the Lenten rose (*H. orientalis* Lamarck). Recently, some hybrid cultivars (*H.* × *hybridus*) between different species of *Helleborus* have been derived. Beruto et al. [2013] showed that *Helleborus* had gained increasing importance in floricultural production. In the Netherlands, during the period 2007–2009, about 1.5 million cut flowers and 500 thousand pot plants of *Helleborus* were sold [Beruto et al. 2013].

The *H. 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 across), which change from white to green after fertilisation [Salopek-Sondi and Magnus 2007]. This species is widely distributed in Southern and Central Europe [Šušek et al. 2005]. *H. niger* is a variable taxon, but only two subspecies are generally recognised: *H. niger* ssp. *niger* and *H. niger* ssp. *macranthus* [Mathew 1989]. The Christmas rose is important

in commercial horticultural production as a garden perennial, blooming in winter and early spring, and as a pot plant and cut flowers. It is one of the most popular cut flowers at Christmas in France, Germany, the Netherlands, Switzerland, and Belgium [Poupet et al. 2006].

The majority of the hellebores grown as *H. orientalis* (Lenten rose) are hybrids of this species and several other *Helleborus* species [Rice and Strangman 1993]. These were previously called *Helleborus orientalis.* Now, they have the new botanical name of *Helleborus × hybridus* [Mathew 1989, Rice and Strangman 1993]. The Lenten rose is an easy to cultivate, hardy, long-lived and evergreen plant tolerant of dry conditions. The flowers come in many shades, are up to 7.5 cm across, and generally in nodding forms; however, upward-facing forms are occasionally found [Burrell and Tyler 2006]. The Lenten rose accepts a wide range of soil conditions, but prefers to have good drainage in the winter. It grows in alkaline, semi-shade conditions in its native habitat [Mathew 1989].

The other species or hybrids are also popular as garden perennials: *H. viridis*, *H. lividus*, *H. foetidus*, *H. purpurascens*, *H. argutifolius*, *H. × nigercors* and *H. × ballardiae.* The flowers of the wild species are large, attractive, and come in various shades of purple, green, and white [Mathew 1989].

Hellebores in natural medicine

Over the past few years, *Helleborus* sp. has become a subject of interest for phytochemistry, pharmacology and other medical research areas because interesting biological activities have been reported for some species. There have been many studies which strongly support the view that extracts of the plants belonging to this genus have beneficial therapeutic effects [Maior and Dobrota 2013]. In the Balkan area, *Helleborus* extracts have long been used in traditional medicine as painkillers and anti-inflammatory remedies, and in veterinary medicine against infectious diseases [Maior and Dobrota 2013]. Several active compounds, including cardioactive glycosides, sterol saponosides, ecdysteroids and γ-lactones, have recently been isolated from plants of this genus. Many other active compounds have been reported and considered to be promising remedies for serious diseases such as cancer, ulcers, diabetes as well as common medical problems such as toothache, eczema, low immunity and arthritis [Maior and Dobrota 2013]. In addition, cytotoxic and apoptosis-inducing effects on leukaemia cells have been described [Jesse et al. 2009]. In Germany, *H. niger* is used in homeopathy and as an adjuvant therapy in the treatment of tumour patients in anthroposophical medicine [Büssing and Schweizer 1998]. An original patent medicine utilizing purified *Helleborus* extracts with antalgic and antirheumatic activities has been formulated in Romania with the registered name Boicil (US Patent) [Kerek 1981]. *H. foetidus* exhibits poisonous and anti-insect activity [Pascual-Villalobos and Robledo 1998].

Harvesting wild *Helleborus* plants is nonproductive because many species are rare and endangered. For this reason, the development of an *in vitro* protocol will be of great importance for the production of planting material to conserve the species and to offset the pressure on the natural population. *In vitro* techniques, consisting of micropropagation, callus culture, cell suspension culture or somaclonal variation, could improve the production of secondary metabolites.

Propagation

Hellebores have been propagated by seed and division. Propagation by cuttings is not an option for the acaulescent *Helleborus* species [Rice and Strangman 1993]. Generative propagation is limited because the seeds require several months to germinate after dispersing from the parent plants. The seeds of *H. niger* have a deep, simple, morphophysiological dormancy caused by the combination of rudimentary embryos and a physiological dormancy that can be broken by cycles of warming and chilling [Niimi et al. 2006]. Therefore, generative propagation requires special conditions, and it takes time to derive plants with a high degree of variation. Vegetative propagation is necessary to maintain the desirable characteristic 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 [Rupprecht and Miessner 1985].

Micropropagation

The increased horticultural and pharmacological interest in *Helleborus* requires the development of efficient protocols for the propagation *in vitro* of hellebore species and cultivars. Although some research on tissue culture has been published, effective commercial micropropagation of these species has not been attained because cultivation *in vitro* is still very difficult [Lim and Kitto 1995, Seyring 2002, Poupet et al. 2006, Dhooghe and Van Labeke 2007, Beruto and Curir 2009, Beruto et al. 2013, Orlikowska et al. 2013, Gabryszewska 2014, 2015, Caesar 2015]. These studies have provided the initial results or protocols for *in vitro* propagation of several *Helleborus* species, such as *H. niger*, *H. argutifolius*, *H. foetidus*, *H. orientalis*, *H. purpurascens*, *H.* × *nigercors* and *H.* × *ballardiae.* However, better protocols are needed to develop an efficient mass production system that will result in increased multiplication and rooting rates, and higher survivability. The efficacy of hellebore micropropagation (multiplication and rooting *in vitro* and acclimatization *ex vitro*) is influenced by several factors, such as: type of initial explants, genotype, growth regulators, and environmental factors (temperature, sucrose, nitrogen salts) [Seyring 2002, Poupet et al. 2006, Dhooghe and Van Labeke 2007, Beruto and Curir 2009, Beruto et al. 2013, Gabryszewska 2013, 2014, 2015, Orlikowska et al. 2013, Caesar 2015, Caesar and Adelberg 2015, Matysiak and Gabryszewska 2016]. Propagation *in vitro* has the advantage of producing identical plants, but some hellebore species, such as *Helleborus* × *hybridus,* have proven to be difficult to produce profitably [Burrell and Tyler 2006].

Initiation of aseptic culture

The choice of explants for the initiation of aseptic culture is very important. Explants with vegetative meristems are often suitable for enhanced axillary branching. The axillary buds present at the base of leaves of various species of hellebores (*H. argutifolius*, *H. foetidus*, *H. niger*, *H. orientalis*, *H.* × *nigercors*, *H. purpurascens*) have been used successfully for initiation *in vitro* [Dhooghe and Van Labeke 2007, Beruto et al. 2013, Gabryszewska 2013, 2014, 2015]. Hellebore cultures have also been initiated from the apical shoot tips (*H.* × *nigercors* and *H.* × *ballardiae*) or from meristem tips (*H. niger*) of seedlings or adult plants [Seyring 2002, Poupet et al. 2006, Caesar 2015]. The Murashige and Skoog [1962] medium or modifications of it, enriched with various growth regulators, have been commonly used for the initial growth of hellebore explants. For example, axillary buds of a few species of hellebores (*H. argutifolius*, *H. foetidus*, *H. niger*, *H. orientalis*) were established on the MS medium supplemented with 2iP and BAP [Dhooghe and Van Labeke 2007]. Primary explants of *H.* × *nigercors* were cultivated on the MS medium supplemented with BAP, 2iP and NAA [Beruto et al. 2013]. The initial growth of *H. purpurascens* and *H. niger* axillary buds and the subsequent subcultures of shoots were performed on the MS medium containing cytokinins (2iP, BAP, kinetin) and GA_3 [Gabryszewska 2014, 2015]. Poupet et al. [2006] produced virus-indexed *H. niger* plants only from the apical meristems on a medium containing kinetin, GA_3, and IBA. However, the use of other basic media has also been reported. Seyring [2002] used the U-medium [Haensch 1999] supplemented with BAP and NAA to establish the initial culture from apical buds of *H. niger* seedlings. This medium was created by Haensch as a universal medium to meet the average nutritional requirements of many different plants. Isolated axillary buds of *H.* × *nigercors* and *H.* × *ballardiae* have been grown on WPM medium with TDZ [Caesar 2015].

Shoot multiplication

Axillary bud development has proven to be the most frequent method applied for the propagation *in vitro* of various hellebore species [Lim and Kitto 1995, Seyring 2002, Poupet et al. 2006, Dhooghe and Van Labeke 2007, Beruto and Curir 2009, Beruto et al. 2013, Gabryszewska 2014, 2015]. Shoot branching is the process by which axillary buds (dormant), located in the axil of a leaf, develop and form new branches (axillary shoots). The interaction of exogenous or endogenous plant hormones and environmen-

tal signals (temperature, light, sugar, nitrogen) regulate axillary bud outgrowth.

During the multiplication phase, the formation of axillary shoots in various *Helleborus* species was achieved by using cytokinins added alone or in combination with auxins or gibberellins. Lim and Kitto [1995] reported a positive effect of BAP (5 mg l^{-1}) on axillary shoot branching in *H. orientalis*, but there was no effect of GA_3 (1 mg l^{-1}) or TDZ (0.1, 1 mg l^{-1}). Similarly, in the case of *H. niger*, BAP added alone to the medium activated axillary shoot proliferation [Poupet et al. 2006]. The combination of cytokinins (2iP 2 mg l^{-1} and BAP 5 mg l^{-1}) with 0.1 mg l^{-1} NOA stimulated axillary shoot growth in *H. niger H. argutifolius*, *H. foetidus* and *H. orientalis* [Dhooghe and Van Labeke 2007]. The basic MS medium supplemented with 1 mg l^{-1} BAP, 2 mg l^{-1} 2iP and 0.1 mg l^{-1} NAA enhanced axillary shoot multiplication in *H.* × *nigercors* [Beruto et al. 2013]. The effects of different concentrations (1 μM – 9 μM) of cytokininis and auxins (BAP, TDZ, BAP + 2iP, BAP + IAA, kinetin + IAA), and basic salts (MS, WPM) were investigated in the multiplication phase in *H.* × *nigercors* and *H.* × *ballardiae* [Caesar 2015]. There were no significant differences between the multiplication ratios of shoots grown on media with the different growth regulators and various basic salts.

The combination of cytokinins and gibberellins in the medium has been found to increase the multiplication rate of axillary shoots of *H. niger* and *H. purpurascens* [Seyring 2002, Gabryszewska 2014, 2015]. The propagation rate of *H. niger* individual seedlings varied widely on the U-medium with 2.2 μM (0.5 mg l^{-1}) BAP and 2.9 μM (1 mg l^{-1}) GA_3 [Seyring 2002]. The various cytokinins (2iP, BAP and kinetin – each at a concentration of 1.0 mg l^{-1}) and 2.5 mg l^{-1} GA_3 added to the MS basal medium stimulated the outgrowth of axillary shoots and their proliferation in the case of *H. purpurascens* and *H. niger* [Gabryszewska 2013, 2014, 2015]. The highest multiplication rate of axillary shoots (2.3) of *H. purpurascens* was found at a temperature of +15°C, on a medium supplemented with sucrose at 30 g l^{-1} and a mixture of cytokinins (2iP, BAP, kinetin) and GA_3, or at a temperature of +20°C on the same medium but with IBA [Gabryszewska 2014]. The multiplication rate of *H. niger* shoots was strongly dependent on the sucrose/nitrogen salt relationship in the medium [Gabryszewska 2015]. The highest multiplication rate of axillary shoots (3.7) was found at a temperature of 15 or 20°C, on a medium with cytokinins and GA_3 supplemented with sucrose at 20–30 g l^{-1} and nitrogen salts at 50%. Also, differences in leaf shape and size as well as in the developmental stage of *H. niger* shoots were observed on the media with different sucrose/nitrogen salts ratios and temperatures (+15, +20°C) [Gabryszewska 2015]. The effect of temperature (18–22°C; 15–17°C; 10–13°C) on the multiplication rate of three hybrid clones was investigated by Caesar [2015]. The highest multiplication rates for *H.* × *hybridus*, *H.* × *nigercors* and *H.* × *ballardiae* (1.45, 1.77 and 2.00, respectively) were at a temperature of 10–13°C.

Various factors (sucrose, phosphate (PO_4^{3-}), nitrate (NO_3^-), ammonium (NH_4^+), micronutrient dilution, TDZ, plant density) and their interaction affected the multiplication rate and plant quality of *H.* × *nigercors* and *H.* × *ballardiae* 'Raulston Remembered' [Caesar 2015, Caesar and Adelberg 2015]. Sucrose (10–30 g l^{-1}) was the most significant factor, which increased the multiplication rate at higher concentrations. The multiplication rate was also strongly influenced by the interaction of sucrose with phosphorus. A high supply of phosphate (6.25 mM) in the medium with a low level of sucrose strongly inhibited multiplication. The best results in multiplication were obtained when both sucrose (30 g l^{-1}) and phosphorus (6.25 mM) were at the highest levels. Also nitrate (NO_3^-), added to the medium as ammonium nitrate and potassium nitrate, significantly affected the multiplication ratio in both hellebore species. The nitrate levels (40 mM) in the Murashige and Skoog [1962] medium were near optimal for the multiplication and quality of hellebore microplants. Consequently, based on these results, the highest multiplication rates for *H.* × *nigercors* (2.31) and for *H.* × *ballardiae* 'Raulston Remembered' (2.62) were obtained on the media with the maximum concentrations of sucrose (30 g l^{-1}) and phosphorus (6.25 mM), and moderate levels of nitrate (40–50 mM) [Caesar 2015, Caesar and Adelberg 2015].

A B C

Fig. 2. Overview of the *Helleborus* species during the multiplication stage: *Helleborus niger* (A), *Helleborus purpurascens* (B) and *Helleborus* × *hybridus* (C) (six weeks after transferring on the multiplication medium). Photo: E. Gabryszewska

Shoots were propagated on media supplemented with sucrose at 20 g l^{-1} and KNO_3 + NH_4NO_3 (50% relative to the MS medium) and a mixture of cytokinins (2iP, BAP and kinetin – each at a concentration of 1.0 mg l^{-1}) with GA_3 at 2.5 mg l^{-1}, and at a temperature of 15°C

It has been reported that increased levels of sucrose in the medium stimulated the accumulation of starch in *H. purpurascens* and *H. niger* shoots propagated *in vitro* [Gabryszewska and Węgrzynowicz-Lesiak 2015]. Analysis of carbohydrate content during the multiplication stage of hellebores revealed that starch was the major carbohydrate accumulated in the shoots. Monosaccharides (glucose and fructose) were present at significantly lower levels, and sucrose was not detected in the shoots of either hellebore species propagated *in vitro*. In general, the shoots of *H. purpurascens* accumulated more starch compared to those of *H. niger*. The starch content was correlated with the multiplication rate and physiological stage of hellebore shoots. The highest concentration of sucrose (80 g l^{-1}), which strongly stimulated starch accumulation, also inhibited the growth of axillary shoots and simultaneously inducted the dormancy of axillary buds [Gabryszewska and Węgrzynowicz-Lesiak 2015]. *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 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. The additional carbon amount supplied from floral photosynthesis has been observed for *H. viridis* and *H. niger* hellebore plants which showed marked juvenile traits [Aschan et al. 2005, Salopek-Sondi and Magnus 2007].

The genotype-dependence of the multiplication rates of some *Helleborus* species (*H. argutifolius*, *H. foetidus*, *H. niger*, *H. orientalis*, *H. purpurascens*, *H.* × *nigercors*, *H.* × *ballardiae*, *H.* × *hybridus*) and selected clones of *H. niger* has already been observed (fig. 2 A–C) [Seyring 2002, Poupet et al. 2006, Dhooghe and Van Labeke 2007, Gabryszewska 2014, 2015, Caesar 2015]. Depending on the *H. niger* genotype, individual seedlings developed from 6 to 280 proliferating shoots during a period of 26 months (average propagation rate was 2.1 per 3-month propagation passage) [Seyring 2002]. Dhooghe and Van Labeke [2007] found that the multiplication rate was significantly higher for *H. niger* than for *H. argutifolius*, *H. foetidus*, and *H. orientalis*.

The light spectrum (more specifically the red, blue, red + blue and white LED and white TL light) had no effect on the propagation rate of *H. orientalis*, but influenced plant morphology *in vitro* [Dhooghe and Van Labeke 2012]. Also Caesar [2015] stated that the quality of light (LED's blue wavelength + red wavelength) did not have a significant effect on the multiplication rate of *H.* × *hybridus*, *H.* × *nigercors* and *H.* × *ballardiae*.

The establishment and multiplication of aseptic culture is one of the major problems associated with micropropagation of hellebores. In the multiplication stage of *Helleborus* × *nigercors*, the bacteria resistant to surface sterilization were isolated and Gram stained [Caesar 2015]. Contamination is not always visible during the initial stage of explants and may become evident after a few subcultures of multiplication. The major genera found as endogenous bacteria in *Helleborus* × *nigercors* shoot culture were: *Paeni-*

bacillus, *Luteibacter*, *Stenotrophomonas*, *Lysobacter and Pseudomonas.* Antibiotic treatment (streptomycin sulfate, gentamicin sulfate, cefotaxime) was unsuccessful at eliminating these contaminants due to severe phytotoxicity associated with high antibiotic concentrations [Caesar 2015].

The multiplication rate of hellebores decreases after about 12–15 months of culture and a re-establishment of new cultures is necessary [Beruto et al. 2013]. It has also been reported that the phase of *in vitro* multiplication can markedly affect the efficiency of subsequent rooting of *Helleborus* plantlets. The use of kinetin in the multiplication medium enhances subsequent rooting, while BAP at high concentration decreases the rooting percentage [Beruto and Curir 2009]. Hellebore shoots multiplied on MS macroelements showed better rooting efficiency compared to those propagated on Quorin and Lepoivre macroelements [Beruto et al. 2013].

In vitro or ex vitro rooting and acclimatization of microplants

A few published reports on *in vitro* propagation of *Helleborus* note that rooting and acclimatization of microplants is not yet problem-free [Beruto et al. 2013]. The rooting rate *in vitro* or *ex vitro* and the acclimatization of microplants are influenced by several factors, such as: species, genotype, growth regulators and environmental factors (temperature, sucrose/nitrogen salts ratio) [Lim ana Kitto 1995, Seyring 2002, Poupet et al. 2006, Dhooghe and Van Labeke 2007, Beruto and Curir 2009, Beruto et al. 2013, Gabryszewska 2014, 2015, Caesar 2015].

Media supplemented with 4.9–29.8 µM (1–6 mg l^{-1}) of IBA or 4.9–29.8 µM (0.9–5.5 mg l^{-1}) NAA displayed significantly different rooting properties in *H. niger* shoot culture [Seyring 2002]. The highest rooting rates (96.4%) and good root formation were recorded on the media with 4.9 µM of IBA (1 mg l^{-1}). The rate of survival of microplants during acclimatization was about 80%. Poupet et al. [2006] demonstrated that various auxins (IAA, IBA, NAA) did not work well and explants of *H. niger* only formed root calli, but better root formation (more than 96%) was induced using various concentrations of IAA in two rooting phases. In the first step (induction of root meristems), shoots were placed on a medium with a low IAA – 1.12 µM (0.2 mg l^{-1}) concentration and kept in darkness at a temperature of 16°C for 2 weeks. In the second step (root development), the microplants were transferred onto a medium with a high level of IAA – 5.6 µM (1 mg l^{-1}). Acclimatization rates of the microplants reached 90% [Poupet et al. 2006].

Drenching of *H. niger* or *H.* × *nigercors* shoots for 1 week in a solution of 3 mg l^{-1} IBA and 1 mg l^{-1} NAA at 5–7°C, and then transplanting them onto the MS medium without hormones positively influenced rooting *in vitro* (87%) and the survival of microplants *ex vitro* (85%). A chilling treatment enhanced the rooting efficiency by 5%. The rooting rate of *H.* × *nigercors* shoots on a solidified MS medium supplemented with 30 g l^{-1} sucrose, 1 mg l^{-1} NAA and 3 mg l^{-1} IBA reached about 80% [Beruto and Curir 2009, Beruto et al. 2013].

The rooting ability of *H. purpurascens* and *H. niger* has been found to be strongly dependent on the sucrose or sucrose/nitrogen salt relationship in the MS media [Gabryszewska 2014, 2015]. In the case of *H. purpurascens*, sucrose at concentrations of 30 and 50 g l^{-1} stimulated the rooting rate (90% rooted shoots) and increased the number of roots per microplants (4.0–4.6) on a medium with 1 mg l^{-1} IBA + 0.1 mg l^{-1} NAA (fig. 3 A). Low (10 g l^{-1}) and high (70 g l^{-1}) levels of sucrose inhibited root formation. During the acclimatization stage, the survival rate of *H. purpurascens* microplants was low [Gabryszewska 2014]. In the rooting stage of *H. niger*, sucrose at a concentration of 50 g l^{-1} strongly stimulated the number of roots per microplant (5.8–6.0) on media with 1 mg l^{-1} IBA + 0.1 mg l^{-1} NAA, in the presence of a reduced level of nitrogen salts (25 and 50% relative to the MS medium) when the temperature was 20 and 15°C, respectively (fig. 3B). The microplants of *H. niger* rooted on media with increased concentrations of sucrose and a low supply of nitrogen salts (25–50%) showed an acclimatization rate from 82 to 100%, and more plants survived when the temperature during rooting *in vitro* was 15°C. The survival rate of microplants rooted on media with the highest nitrogen salts content (100% relative to the MS medium) depended on the concentrations of sucrose in the medium (10–80 g l^{-1}) and

A

B

Fig. 3. Overview of the Helleborus purpurascens (A) and Helleborus niger (B) in the rooting stage

Shoots of the Helleborus purpurascens were rooted on the media supplemented with sucrose 30 g l-1 and KNO3 + NH4NO3 (50% according to the MS medium) at temperature +20°C and Helleborus niger shoots rooted on the media with 70 - 80 g l−1 sucrose and KNO3, NH4NO3 (100% according to the MS medium) at temperature +20°C. For rooting purposes, the single shoots were on the media with 50% or 100% of nitrogen salts (according to the MS medium – KNO3, NH4NO3) supplemented with IBA 1 mg l-1 and NAA 0.1 mg l-1

varied from 0 to 100% [Gabryszewska 2015]. The maximal photochemical efficiency of photosystem II (PSII) immediately upon removal of *H. niger* microplants from *in vitro* culture ranged from 0.24–0.81 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 acclimatization *ex vitro* and further growth of plants. 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 acclimatization *ex vitro* of *H. niger* plantlets. High-quality microplants derived from such conditions more easily adapt to *ex vitro* conditions and exhibit faster growth in the natural environment [Matysiak and Gabryszewska 2016]. After 2–3 months, young plants of *H. niger* and *H. purpurascens* were ready to grow under regular growing condition (fig. 4 A, B). The first flowering of *H. niger* was after 1 year from the *ex vitro* establishment of microplants in the greenhouse (fig. 5).

A

B

Fig. 4. Young plants (six months old) of *Helleborus niger* L. (A) and *Helleborus purpurascens* Waldst. et Kit. (B) propagated *in vitro*. Photos: E. Gabryszewska

Dhooghe and Van Labeke [2007] suggested an *ex vitro* rooting method for four *Helleborus* species, which were induced for one week by being drenched in a solution of IBA (3 mg l^{-1}) and NAA (1 mg l^{-1}) at 5°C, but no data on the rooting rate or survival percentage was provided.

A low survival percentage (50–56%) was obtained in *ex vitro* rooting of *H. orientalis* [Lim and Kitto 1995].

Red LED light stimulated the number of roots on *H. orientalis* micro-shoots according to Dhooghe and Van Labeke [2012]. They also found that red and white LED lights, as well as control TL lamps, contributed to the formation of a significantly longer root compared to blue and red + blue LED lighting. Rooting responses of two hybrid clones *H.* × *nigercors* and *H.* × *ballardiae* to the consistency of the medium (gellan, liquid) and changes in light quality (monochromatic LEDs: 100% red, 100% blue, 33.3% red + 66.7 blue, 66.7 red + 33.3 blue) were investigated by Caesar [2015]. Microshoots were grown at 12°C on the MS medium with 5.4 µM (1 mg l^{-1}) NAA and 30 g l^{-1} of sucrose at a 16-hour photoperiod. Rooting rates varied from 6.7 to 86% for *H.* × *ballardiae* and from 0 to 95% for *H.* × *nigercors.* Light quality did not have a significant effect on the rooting percentage, but plants grown on the media with 3 g l^{-1} gellan showed significantly higher rooting percentages [Caesar 2015].

The results presented by Orlikowska et al. [2013] showed that inoculation of *H. niger* shoot cultures with the bacteria *Burkholderia phytofirmans* stimulated root formation. The best rooting rate (100%) was obtained for shoots which had been induced for root formation on a medium with 3 mg l^{-1} IBA and 1 mg l^{-1} NAA, and then inoculated with *B. phytofirmans*. This bacterial species produces indole-3-acetic acid (IAA) and shows strong plant growth-promoting effects [Weilharter et al. 2011]. Lower rooting rates of *H. niger in vitro* were found in the shoots inoculated with *B. phytofirmans* (95%) and in the shoots growing on media with 3 mg l^{-1} IBA and 1 mg l^{-1} NAA (94%), or without auxins (83%). There is no information on the survivability of rooted shoots in *ex vitro* conditions [Orlikowska et al. 2013].

Fig. 5. The first flowering of *H. niger* plants after 1 year from the acclimatization *ex vitro* of microplants. Photo: E. Gabryszewska

The micropropagated plants of *Helleborus niger* were inoculated with arbuscular endomycorrhizal fungi or/and *Agrobacterium radiobacter*. The inoculation with *A. radiobacter* had significantly positive effect on growth and development of plants multiplicated by *in vitro* techniques as compared to the dual inoculation [Susek et al. 2010].

Tissue culture in hellebore breeding

In response to the increased horticultural interest in *Helleborus*, breeding programs are currently focusing on increasing genetic variation.

Interspecific hybridisations

Interspecific hybridisations have been carried out in many crop plants to increase genetic variation. *H. niger* and *H.* × *hybridus*, a group of hybrids that have *H. orientalis* as one parent in common, have attained the highest commercial interest. Observations based on existing *Helleborus* hybrids with regard to hybridisation ability have been made [Mathew 1989]: various fertile interspecific hybrids exist within the section Helleborastrum, while the inter-sectional hybrids *H.* × *ballardiae* and *H.* × *nigercors* (hybrids between *H. niger* and the stemmed species *H. lividus* and *H. argutifolius*, respectively) produce intermediate but sterile phenotypes [Meiners and Winkelmann 2012]. Some traits of other species, for example flower colour, growth type, scent and disease tolerance, are valuable for the horticultural improvement of hellebores and which should be introgressed into *H.* × *hy-*

bridus or *H. niger* through interspecific hybridisation [Meiners et al. 2011]. However, pre- and post-zygotic barriers often hinder the development of interspecific hybrids. Crossing barriers in *Helleborus* were localised as predominantly post-zygotic according to *in situ* pollen tube staining with aniline blue [Meiners and Winkelmann 2012]. For certain crosses, pre-zygotic barriers could also be assumed, but pollen tube growth was not totally inhibited. Therefore, embryo rescue techniques via ovule culture were established to overcome the post-zygotic barriers.

Polyploidization of Helleborus species *in vitro*

Polyploidization is a prominent method to induce variation in plants. Polyploids can be induced by two mechanisms: mitotic polyploidization, which is based on the doubling of somatic tissues, and meiotic polyploidization, which generates 2n gametes [Ramsey and Schemske 1998]. Recently, it has been demonstrated that polyploidy is not simply genome duplication, but it results in a whole spectrum of molecular and physiological modifications [Adams and Wendel 2005]. Chromosome doubling *in vitro* can be induced by several antimitotic agents. The most commonly used are colchicine, oryzalin and trifluralin. The induction step *in vitro* depends on a large number of variables: media, antimitotic agents, explant types, exposure times and concentrations [Dhooghe et al. 2011].

All of the *Helleborus* species are diploid and have a chromosome number of 2n = 32 [Benett and Smith 1976, Zonneveld 2001]. Dhooghe et al. [2009] investigated the effect of antimitotic agents (colchicine, oryzalin, trifluralin) on chromosome doubling in shoot culture of *Helleborus niger*, *H. orientalis* and *H.* × *nigercors*. The tetraploids were obtained for *H. niger* using oryzalin or trifluralin. In the case of *H.* × *nigercors,* only trifluralin induced polyploidization. No tetraploids were induced for *H. orientalis.* In all *Helleborus* species, colchicine was ineffective for chromosome doubling [Dhooghe et al. 2009].

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