Journal of Horticultural Research 2025, vol. 33(2): 41–58
DOI: 10.2478/johr-2025-0016

EFFECT OF MEDIUM COMPOSITION AND CULTURE CONDITIONS ON THE *IN VITRO* MULTIPLICATION, ROOTING AND ACCLIMATIZATION OF *HELLEBORUS* 'MOLLY'S WHITE' AND 'ANNA'S RED'

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Received: June 2025; Accepted: November 2025

ABSTRACT

The genus *Helleborus* belongs to the family Ranunculaceae and comprises about 22 species, which are distributed across Europe and West Asia. Hellebores are popular as garden perennials, pot plants, cut flowers, and medicinal plants. The present study aimed to evaluate the effects of sucrose level (20, 30, 40**,** and 50 $g \cdot L^{-1}$), mineral salts content (50%, 100%, 150% of Murashige and Skoog 1962 medium – MS), growth regulators (BAP – 6-benzylaminopurine, 2iP – 6-(γ,γ-dimethylallylamino) purine, kinetin, GA_3 – gibberellic acid, IBA – indole-3-butyric-acid), and white light sources (fluorescent and LED) for multiplication and *in vitro* rooting *Helleborus* of 'Molly's White' and 'Anna's Red'. Additionally, the post-effect of white, blue, and red light (LED) used during the storage of shoots at a temperature of 15°C for 3 months in glass jars and plastic vessels on the subsequent *in vitro* rooting and on *ex vitro* acclimatization was investigated. The results presented here showed that sucrose and nitrogen salts were more critical for axillary shoot branching and subsequent *in vitro* rooting in both hellebore genotypes than the concentration of growth regulators in the multiplication and rooting media. Low level of sucrose (20 $g \cdot L^{-1}$) and standard content of mineral salts (100% in the MS medium) were optimal for the development of axillary shoots. The medium composition used in the multiplication experiments markedly affected the rooting ability of both cultivars. The sucrose/nitrogen salts ratio in the multiplication medium was more critical for subsequent rooting than the type and concentration of growth regulators or the composition of the rooting media. Genotype 'Molly's White' exhibited the best rooting after multiplication on the medium with high (40 $g \cdot L^{-1}$) and low (20 $g \cdot L^{-1}$) sucrose levels, but 'Anna's Red' only on the medium with low sucrose content (20 $g \cdot L^{-1}$). The white LED increased the multiplication and rooting rates of both hellebores compared with white fluorescent light. Plastic vessels significantly increased root number in both hellebore genotypes. The post-effect of white, blue, and red light (LED) used during the storage of cultures had a weak influence on *ex vitro* acclimatization compared to *in vitro* rooting. Genotypic differences in multiplication rate, rooting ability, and *ex vitro* acclimatization efficiency were observed.

Key words: *in vitro* propagation, sucrose, mineral salts, LED

INTRODUCTION

The genus *Helleborus* belonging to Ranunculaceae family includes about 22 species (Meiners et al. 2011). The majority of *Helleborus* species are naturally widely distributed over various parts of Europe, with only one species (*H. thibetanus*) native to western China. *Helleborus* is a winter or early spring flowering perennial. It can be used as a garden or pot plant, and some species are also grown as cut flowers (e.g., *H. niger* and *H. hybridus*). More than 1,000 novel *Helleborus* hybrids and cultivars have been obtained by interspecific hybridization. The economic value of the genus *Helleborus* lies not only in its ornamental value but also in its therapeutic benefits. Several studies have shown that *Helleborus* extracts are a potentially valuable source for medical uses (Long et al. 2023). The increased horticultural and pharmacological interest in *Helleborus* necessitates the development of efficient protocols for the *in vitro* propagation of hellebore species and cultivars. Although studies on tissue culture have been published, effective commercial micropropagation of these species has not been achieved because *in vitro* propagation of some hellebore species or hybrids remains very difficult. During commercial *in vitro* propagation of hellebores, the success of micropropagation is highly dependent on species, cultivar, and clone.

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Helleborus plants are reproduced by both generative (seeds) and vegetative (*in vivo* division of rhizomes and micropropagation). It has been reported that rhizome division propagation has a low multiplication rate and is a time-consuming process. Vegetative propagation is necessary to maintain the desirable characteristics of a particular hellebore cultivar. *In vitro* propagation is a promising method for propagating hellebores. Some studies on *in vitro* propagation of *Helleborus* have presented that the shoot multiplication efficiency, rooting ability and *ex vitro* survival are influenced by several factors, including genotype, growth regulators, sucrose, phosphate (PO_4^{3-}), nitrate (NO_3^-), ammonium (NH_4^+), micronutrient dilution, sugar/nitrogen ratio, sucrose/phosphate interaction, inoculation plants with bacteria, and environmental factors (temperature, type of light) (Seyring 2002, Poupet et al. 2006, Dhooghe & Van Labeke 2007, Beruto & Curir 2009, Beruto et al. 2013, Orlikowska et al. 2013, Gabryszewska 2014, 2015, 2017, Caesar 2015, Caesar & Adelberg 2015, Matysiak & Gabryszewska 2016). Some *Helleborus* species and cultivars are quite recalcitrant to micropropagation. This method is not yet free of difficulties, including a long *in vitro* initiation time, a low multiplication rate, complicated rooting, and numerous problems during acclimatization.

The Murashige and Skoog (MS) standard medium (1962) and a sucrose concentration of 30 $g \cdot L^{-1}$ are typically used for the *in vitro* propagation of plants. However, optimal sugar and mineral salt concentrations vary among plant species, genotype, or developmental phase and require adjustment. In the case of *Billbergia zebrina* micropropagation, the 200% MS salt concentration in the liquid medium resulted in the highest shoot number. An increase in sucrose level in the medium hurt the chlorophyll a, b, and carotenoid contents. Conversely, the content of total soluble sugars in *B. zebrina* shoots increased with increasing sucrose concentrations. Additionally, a high sucrose concentration (45 and 60 $g \cdot L^{-1}$) induced greater root numbers (Martins et al. 2015). A high concentration of sucrose in the medium has been detrimental for the *in vitro* photosynthetic activity of strawberry (Hdider & Desjardins 1994). In the case of *Helleborus niger*, a high concentration of sucrose (50 $g \cdot L^{-1}$) and nitrogen salts reduced to 25% in the MS medium decreased the photochemical efficiency of microplants in *ex vitro* conditions (Matysiak & Gabryszewska 2016).

The two most abundant nutrients in plant cells *in vivo* and *in vitro* are carbon (C) and nitrogen (N). They are also crucial for plant growth and development as essential signalling molecules that regulate various physiological processes. Sugars regulate plant growth and *in vitro* development by acting as both an energy/carbon source as well as a critical signalling molecule, influencing multiple physiological processes such as cell division and expansion, and development under *in vitro* conditions. Molecular analyses have revealed extensive interactions between sugar and plant hormone signalling, and a central role for hexokinase as a glucose sensor (Shah et al. 2019). Sugars have been postulated to help regulate the synthesis, conjugation, or transport of gibberellins and abscisic acid. On the contrary, abscisic acid, gibberellins, and cytokinins have been shown to help regulate sugar metabolism or transport (Gibson 2004). Apart from these functions, sugars act as an osmotic regulator (De Paiva Neto & Otoni 2003). They can play a protective role against stress factors, for example, as osmoprotectants or signalling molecules in the regulation of many defensive reactions (Ciereszko 2018). The C/N ratio, along with C status and N availability, significantly affects plant growth and development. Carbon and nitrogen metabolism is modulated by the interaction of C signalling with N signalling or by C/N ratio signalling. Many developmental processes respond to carbon and nitrogen provisions: leaf growth, root system architecture, and seed development. In some cases, the impact of high C availability on plant development and hormone signalling appears to depend on N level (Coruzzi & Zhou 2001, Zheng 2009, Fañanás-Pueyo et al. 2025).

Light-emitting diodes (LEDs) have recently been proposed as an energy-efficient light source for various applications of plant tissue culture. LED lighting has been found to have positive effects on *in vitro* shoot and root development as well as *ex vitro* acclimatization of plantlets (Nhut et al. 2003, Daud et al. 2013, Hung et al. 2015, Ferreira et al. 2017, Davidović Gidas et al. 2025).

Reduced-temperature storage is a method implemented by gene banks to prolong the intervals between explant transplantations. Storage duration is typically between 9 and 24 months (or longer) (Reed 1999, 2002). The most commonly used method for reducing growth is the cold storage of shoot cultures. Incubation at a temperature lower than that required for optimal growth will reduce metabolic activities, such as respiration, water loss, wilting, and ethylene production. Cold storage is often combined with reduced light intensity or total darkness. The storage of shoot cultures in total darkness

is mainly used by commercial tissue culture laboratories to minimize costs (Benelli et al. 2022). The type of containers used to maximize the storage duration of shoot cultures is a particularly important factor during long-term storage. Several culture containers were tested, including glass jars or tubes, the GA-7 Magenta™ boxes (a polypropylene container), Microbox ECo 2™ (a polycarbonate container), Starpac Pac™, Vitrovent (Benelli et al. 2022, Abacı et al. 2025). The type of container is essential during the transportation of *in vitro* plants in commercial laboratories. Glass containers are heavy and fragile during transportation. Using different types of plastic containers can reduce the risk associated with long-distance shipping of *in vitro* explants.

Nowadays, micropropagation is a technique used by companies to produce large numbers of plants for the international market and as an irreplaceable tool in breeding and research. Various plant species and cultivars present different challenges for micropropagation companies. The development of protocols for *in vitro* propagation is economically interesting only for highly valuable *Helleborus* species or interspecific hybrids (Dhooghe et al. 2018).

For commercial companies, reducing production costs to meet pricing pressure remains the most critical issue. Effective cost reduction strategy can involve optimizing and improving the *in vitro Helleborus* propagation procedure.

This study aims to learn about the relationships between the media components and light source, and finally to propose an optimized protocol for the *in vitro* propagation of the *Helleborus* 'Molly's White' and 'Anna's Red', including the optimal medium composition, light condition, and culture container type to produce microshoots capable of effective rooting and acclimatization.

MATERIALS AND METHODS

Experiments were carried out in the commercial Norwa Tissue Culture Laboratory in Piaseczno (Poland), under commercial conditions.

Plant material

The study was conducted with *Helleborus* 'Molly's White' and 'Anna's Red.' *Helleborus* × *iburgensis* 'Molly's White' (bred at RD Plants in the UK) was derived from hybridization of *Helleborus hybrids* × *H.* × *ballardiae*, and *H. lividus* (Plant Delights Nursery, https://plantdelights.com [15.06.2025]). *Helleborus* 'Anna's Red' was bred by Rodney Davey in the UK. It is the first red flowering hellebore with marbled foliage. It was a surprise success of crossing *H. lividus* × *H. niger*, and *H. hybridus* (Zahradnictví Milan Havlis, https://www.havlis.cz [15.06.2025]).

Multiplication

Stabilized 5-week-old cultures that were propagated on MS solid medium with 0.2 mg·L^{-1} BA, 30 g·L^{-1} sucrose, and 6.5 g·L^{-1} Phytoagar (Duchefa, the Netherlands) were used to set up the experiments. The culture of axillary shoots was subcultured on the fresh medium every 5 weeks.

The first experiment was conducted to assess the effect of MS medium with modified sucrose, mineral salts, and growth regulators (1 MM–8 MM; Table 1) on the axillary shoot number and multiplication rate. The multiplication medium – 1 MM was used as a control. It is a standard medium composition for the multiplication stage of hellebores in the Norwa Plants Laboratory. Each treatment consisted of four glass jars (540 ml, capped with polyethylene caps) with five explants, which were treated as a repeat. The experiment was repeated three times. After 5 weeks, the number of axillary shoots and the multiplication rate were evaluated.

In the second experiment, effect of white LED and fluorescent light (control) on the multiplication rate and growth of *Helleborus* shoots grown on the 1 MM for 4 or 5 weeks was studied. Each treatment consisted of four glass jars (540 ml, capped with polyethylene caps) with five explants. The glass jar with five shoots was a repeat. The experiment was repeated three times. After 4 and 5 weeks, the number of axillary shoots, the multiplication rate, and the fresh weight of shoots were measured.

Storage

Shoots of both hellebore genotypes were stored *in vitro* under white, blue, and red LED in plastic vesseł̷s and glass jars at 15°C for 3 months on the 1 MM (Table 2). After storage, shoots were multiplied in one 5 week passage on the 1 MM, then rooted *in vitro* in plastic vessels and glass jars (5 weeks) on the rooting medium (1 RM) under white LED (Table 3). Each treatment consisted of five glass jars (540 ml, capped with polyethylene caps) with five explants or five plastic vessels (Practipack Polipropylen 500 ml) with ten explants. The experiment was repeated twice.

Rooting

Four experiments were conducted to optimize *in vitro* rooting conditions. The first experiment was aimed to assess the effect of three rooting media – 1 RM–

3 RM (Table 3) on root formation from shoots previously cultivated on different multiplication media – 1 MM–8 MM (Table 2). As a control (Table 3) was used 1 RM [MS supplemented with 30 $g \cdot L^{-1}$ sucrose, 50% of the whole mineral salts of MS medium, and 2 $mg \cdot L^{-1}$ IBA (standard composition for rooting in this laboratory)]. Each treatment consisted of three plastic vessels with 15 explants. The experiment was repeated twice. The percentage of rooted shoots and the quality assessment of root system were analyzed.

In the second experiment, the effect of white fluorescent light (control) and white LED on the rooting of shoots and the growth of plantlets of hellebore 'Molly's White' and 'Anna's Red' was studied. In the experiment, the 1 RM (Table 3) was used. Each treatment consisted of four glass jars with five explants. The experiment was repeated twice. The fresh weight of plantlets, the number of roots, the number of leaves and the quality of root system were assessed.

In the third experiment, the growth characteristics of the 'Molly's White' and 'Anna's Red' plantlets, rooted in plastic vessels and glass jars, were evaluated. In the experiment, the 1 RM (Table 3) was used. Each treatment consisted of four glass jars with five explants or four plastic vessels with ten explants. The glass jar with five explants or plastic vessels with ten explants was a repeat. There were four repetitions. The experiment was repeated twice. The fresh weight of plantlets, the number of roots, the number of leaves and the quality assessment of root system were evaluated.

In the fourth experiment, the post-effect of white, blue, and red light (LED) used during the storage of shoots (15°C for 3 months) in plastic vessels or glass jars on the subsequent *in vitro* rooting was investigated. In the experiment, the 1 RM was used. Shoots were rooted under white LED (Table 2). Each treatment consisted of four glass jars with five explants or four plastic vessels with ten explants as repeats. There were four repetitions. The experiment was repeated twice. The percentage of rooted shoots and the quality assessment of root system were assessed.

***Ex vitro* acclimatization**

The post-effect of white, blue, and red light emitted by LEDs used during the storage of shoots on the survival and acclimatization of 'Molly's White' and 'Anna's Red' was observed. Well-rooted plantlets from the fourth rooting experiment were transferred and planted into a multipot system (Modular Multitray 104, Modiform, the Netherlands) in the greenhouse. Six months after acclimatization, the plantlets were ready for transfer to greenhouse cultivation.

Measurements, observations, and statistical analysis

After finishing multiplication, the number of axillary shoots, the multiplication rate, and the fresh weight of shoots were measured after 4–5 weeks of culturing. In the end of the rooting period (6 weeks), the percentage of rooted shoots, the quality assessment of root system (scale 0–5: 0 – no roots, 1 – very small roots, 2 – small roots, 3 – medium roots, 4 – large roots, 5 – very large roots), the number of roots, the fresh weight of plantlets, the number of leaves and shoot length were analyzed. All experiments were analyzed as one-factorial using ANOVA and Tukey's test to estimate differences between means at the 5% level.

Table 1. Multiplication media (MM) composition used in multiplication experiments

Multiplication medium	Modification of mineral salts composition in MS medium (%)	Sucrose concentration ($g \cdot L^{-1}$)	Growth regulators concentration ($mg \cdot L^{-1}$)
1 MM control	100% MS	30	0.2 BAP
2 MM	100% MS	20	0.2 BAP
3 MM	100% MS	40	0.2 BAP
4 MM	150% MS	30	3.0 BAP, 3.0 2iP, 3.0 kinetin, 5.0 GA_3
5 MM	150% MS	40	3.0 BAP, 3.0 2iP, 3.0 kinetin, 5.0 GA_3
6 MM	150% MS	50	3.0 BAP, 3.0 2iP, 3.0 kinetin, 5.0 GA_3
7 MM	100% MS	20	1.0 BAP, 1.0 2iP, 1.0 kinetin, 2.5 GA_3
8 MM	150% MS	50	3.0 BAP, 3.0 2iP, 3.0 kinetin, 5.0 GA_3

Murashige and Skoog medium
BAP – 6-benzylaminopurine
2iP – 6-(γ,γ-dimethylallylamino) purine
GA_3 – gibberellic acid

Table 2. Light and temperature in the experiments on multiplication, rooting, and storage stages

Source of light	Light wavelength (nm)	Light intensity PPFD (µmol ·m^{-2} sec^{-1})	Temperature (°C)
multiplication			
fluorescent white LED	482.9 575.4	70	20
rooting			
fluorescent white LED	482.9 575.4	70	20
storage			
white LED blue LED red LED	482.9 462.0 656.7	40	15

* IC Bright Technologies Ltd. (Model: T812B02A-22C, T812B01B-24C)
Cultures were grown under a 12 h light cycle

Table 3. Rooting media (RM) composition used in rooting experiments

Rooting medium	Modification of mineral salts composition in MS medium (mg·L^{-1})	Sucrose concentration (g·L^{-1})	Concentration of IBA (mg·L^{-1})
1 RM control	50% of mineral salts composition	30	2
2 RM	50% KNO_3, 50% NH_4NO_3	30	2
3 RM	50% KNO_3, 50% NH_4NO_3	40	2

Murashige and Skoog medium

Table 4. Effect of multiplication media (MM) composition on the multiplication rate (axillary shoots branching) of *Helleborus* 'Molly's White' and 'Anna's Red' grown at 20°C under white fluorescent light

MM	Multiplication rate	
	'Molly's White'	'Anna's Red'
1 MM control	1.13	1.27
2 MM	2.47*	1.90*
3 MM	1.70	1.40
4 MM	1.37	1.13
5 MM	1,27	1.50
6 MM	1.67	1.50
7 MM	2.47*	1.23
8 MM	1.60	1.83*
	LSD 0.7169	*LSD 0.6286*
	SE 0.1463	*SE 0.1283*

The assessment of the significance of differences was performed separately for each cultivar
LSD – Tukey's Least Significant Difference, SE – standard error, * Significant

RESULTS

Multiplication

In the first experiment, the influence of the sucrose concentrations (20–50 g·L^{-1}) and the different levels of mineral salts (100% and 150% of MS medium) as well as the effect of growth regulators (BAP – 6-benzylaminopurine, 2iP – 6-(γ,γ-dimethylallylamino) purine, kinetin, GA_3 – gibberellic acid) on *in vitro* multiplication of *Helleborus* 'Molly's White' and 'Anna's Red' were examined. On the control medium (1 MM), the multiplication rate in 'Molly's White' was the lowest (Table 4), while the highest was on the 2 MM and 7 MM, which contained 20 g·L^{-1} sucrose, 100% KNO_3 and NH_4NO_3. Morover, in the 2 MM, there was only BAP, but in the 7 MM was a mixture of three cytokinins (2iP, BAP, kinetin) with gibberellin (GA_3). In 'Anna's Red', the highest multiplication rate was 1.90 and 1.83 on the 2 MM and 8 MM, respectively. The ability of cultures to produce axillary shoots decreased with the increase of sucrose (40–50 g·L^{-1}) and mineral salts level (150%) in the media. The morphology of *Helleborus* 'Molly's White' and 'Anna's Red', which were grown on the multiplication media containing the best composition (2 MM, 7 MM, 8 MM) is presented in Figure 1.

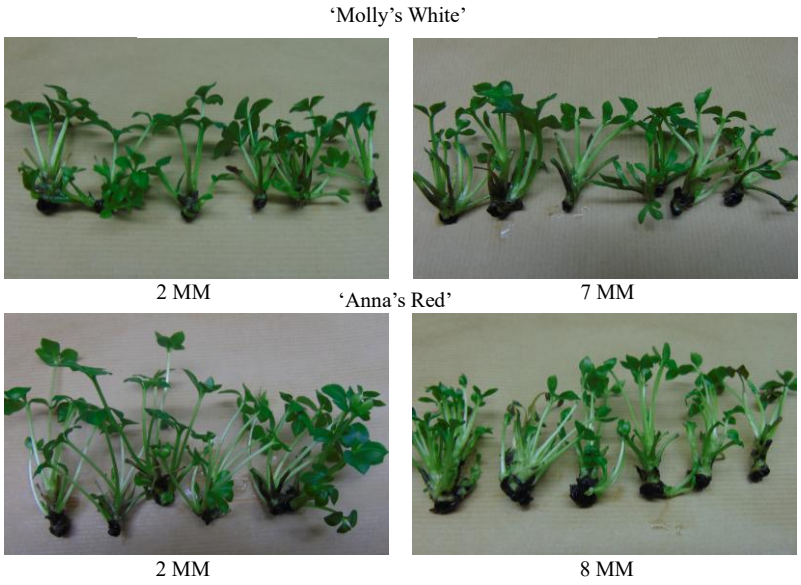

Figure 1. The morphology of *Helleborus* 'Molly's White' and 'Anna's Red' grown on the multiplication media containing the best composition at 20°C under white fluorescent light

The second experiment aimed to compare hellebore shoot growth and branching response under white fluorescent light (control) and white LED (Table 5). A maximum multiplication rate – 2.96, in 'Molly's White' was found for the shoots grown under white LED in 5-week passage, and 1.96 in 4-week passage (Table 5). A similar trend was observed in 'Anna's Red', where multiplication rates were 2.16 and 2.68 at 4-week and 5-week passage, respectively. The longer passage caused an increase in the multiplication rate and in fresh weight in both cultivars. Genotypic differences in shoot growth, fresh weight, and multiplication rate were observed.

Rooting

A first rooting experiment was conducted to assess the post-effect of various multiplication media (1 MM–8 MM) on the quality of the root system and the percentage of rooted shoots. The 'Molly's White' shoots rooted on 1 RM (30 $g \cdot L^{-1}$ sucrose, 50% of mineral salts of the MS, and 2 $mg \cdot L^{-1}$ IBA) that was multiplied on the 2 MM or 7 MM (supplemented with 20 $g \cdot L^{-1}$ sucrose and a standard concentration of nitrogen salts – 100% KNO_3 and 100% NH_4NO_3) produced the highest (4.47 and 4.67) quality root system, regardless of type and concentration of growth regulators during multiplication (Table 6, Fig. 2). Also, 3 MM, with higher concentration of sucrose (40 $g \cdot L^{-1}$) and the 100% of nitrogen salts, strongly affected root formation and their quality (4.47) on 'Molly's White' plantlets. The shoots of 'Anna's Red' rooted on 1 RM produced the highest quality of root system (4.07 and 3.80) after multiplication on 2 MM and 8 MM, which were supplemented with 50 $g \cdot L^{-1}$ sucrose and nitrogen salts level of 150% KNO_3 and 150% NH_4NO_3 (Table 6, Fig. 2).

On the 2 RM (30 $g \cdot L^{-1}$ sucrose, 50% KNO_3, 50% NH_4NO_3, and 2 $mg \cdot L^{-1}$ IBA) both cultivars showed lower rooting capacity. The shoots of 'Molly's White' produced the highest (3.00 and 3.53) quality of root system after multiplication on the 3 MM and 7 MM. However, the differences was not statistically significant compared to control medium (Table 6, Fig. 2). In 'Anna's Red', the best rooting (4.13) was stated in the shoots multiplied on the 2 MM (Table 6, Fig. 2).

The shoots of 'Molly's White' rooted on 3 RM (40 $g \cdot L^{-1}$ sucrose, 50% KNO_3 and 50% NH_4NO_3, and 2 $mg \cdot L^{-1}$ IBA) produced the highest quality root system (4.80 and 3.47) after multiplication on 2 MM and 3 MM (Table 6, Fig. 2). The shoots of 'Anna's Red' multiplied on the 2 MM produced the best root system on the 3 RM (Table 6, Fig. 2).

The rooting percentage of both cultivars varied on the same rooting medium (1 RM–3 RM) depending on the sucrose (20–50 $g \cdot L^{-1}$) and mineral salts (100–150%) concentrations in the multiplication media. The highest percentage of rooted shoots (100%) was closely related to the best root quality (Table 6, Fig. 2). High level of sucrose (40–50 $g \cdot L^{-1}$) and nitrogen salts (150%) in the multiplication media inhibited root formation and their growth on the rooting media in both genotypes.

Table 5. Effect of light type on the number of shoots, multiplication rate and shoot fresh weight of *Helleborus* 'Molly's White' and 'Anna's Red' grown on the multiplication medium (1 MM) for 4 or 5 weeks, at 20°C

'Molly's White'			
Color and source of light	Number of shoots	Multiplication rate	Shoot fresh weight (g)
Passage – 4 weeks			
white fluorescent	1.90	1.13	0.47
white LED	3.28*	1.96*	1.16*
	LSD 0.8058	*LSD 0.4783*	*LSD 0.4053*
	SE 0.2050	*SE 0.1217*	*SE 0.1031*
Passage – 5 weeks			
white fluorescent	1.90	1.13	0.47
white LED	5.04*	2.96*	1.37*
	LSD 0.8004	*LSD 0.4361*	*LSD 0.4229*
	SE 0.2037	*SE 0.1110*	*SE 0.1076*
'Anna's Red'			
Passage – 4 weeks			
Color and source of light	Number of shoots	Multiplication rate	Shoot fresh weight (g)
white fluorescent	1.93	1.27	0.89
white LED	3.21	2.16	0.94
	LSD 2.8022	*LSD 1.7786*	*LSD 0.4672*
	SE 0.7130	*SE 0.4526*	*SE 0.1189*
Passage – 5 weeks			
white fluorescent	1.93	1.27	0.89
white LED	3,97*	2.68*	1.01
	LSD 1.2531	*LSD 0.8137*	*LSD 0.3183*
	SE 0.3189	*SE 0.2071*	*SE 0.0810*

The assessment of the significance of differences was performed separately for each cultivar, passage (4 and 5 weeks) and analyzed feature
LSD – Tukey's Least Significant Difference, SE – standard error, * Significant

Table 6. The post-effect of various multiplication media (MM) on the percentage of rooted shoots and the quality assessment of root system of *Helleborus* 'Molly's White' and 'Anna's Red' grown on the MS rooting media (RM) at 20°C under white fluorescent light

Rooting medium	Multiplication medium	'Molly's White'		'Anna's Red'	
		Percentage of rooted shoots (%)	Quality assessment of root system	Percentage of rooted shoots (%)	Quality assessment of root system
1 RM control	1 MM control	87	3.00	47	0.47
	2 MM	100	4.67*	100	4.07*
	3 MM	100	4.47*	0	0.00
	4 MM	13	0.40	0	0.00
	5 MM	0	0.00	0	0.00
	6 MM	60	1.40	27	0.40
	7 MM	100	4.47*	67	0.67
	8 MM	53	0.40	100	3.80*
			LSD 1.4390		*LSD 0.9662*
			SE 0.3300		*SE 0.2216*
2 RM	1 MM control	100	2.67	80	0.93
	2 MM	47	0.60	100	4.13*
	3 MM	100	3.00	33	0.40
	4 MM	73	0.80	73	0.93
	5 MM	18	0.40	20	0.40
	6 MM	20	0.40	20	0.40
	7 MM	100	3.53	33	0.40
	8 MM	80	0.93	80	1.20
			LSD 1.7185		*LSD 1.0939*
			SE 0.3942		*SE 0.2509*
3 RM	1 MM control	60	0.60	53	0.60
	2 MM	100	4.80*	100	3.40*
	3 MM	100	3.47*	40	0.40
	4 MM	20	0.40	87	0.87
	5 MM	20	0.40	13	0.40
	6 MM	67	0.73	47	0.47
	7 MM	100	2.80	60	0.60
	8 MM	63	0.40	47	0.60
			LSD 1.3017		*LSD 1.1003*
			SE 0.2986		*SE 0.2524*

1 MM – control multiplication medium, 1 RM – control rooting medium (see: Material and methods)
The quality assessment of root system*(scale 0–5: 0 – no roots, 1 – very small roots, 2 – small roots, 3 – medium roots, 4 – large roots, 5 – very large roots
The assessment of the significance of differences was performed separately for each cultivar and each rooting medium (1 RM–3 RM)
LSD – Tukey's Least Significant Difference, SE – standard error, * Significant

‘Molly’s White’
1 RM (control): 30 g·L^{-1} sucrose, 50% MS mineral salts, 2 mg·L^{-1} IBA

1 MM (control) 2 MM 3 MM 4 MM

5 MM 6 MM 7 MM 8 MM

2 RM: 30 g·L^{-1} sucrose, 50% KNO_3, 50% MS NH_4NO_3, 2 mg·L^{-1} IBA

1 MM (control) 2 MM 3 MM 4 MM

5 MM 6 MM 7 MM 8 MM

3 RM: 40 g·L^{-1} sucrose, 50% KNO_3, 50% MS NH_4NO_3, 2 mg·L^{-1} IBA

1 MM (control) 2 MM 3 MM 4 MM

5 MM 6 MM 7 MM 8 MM

Figure 2. Plantlets of *Helleborus* 'Molly's White' and 'Anna's Red' multiplied on various media (1 MM–8 MM) and subsequently rooted on the three different media (1 RM–3 RM) at 20°C under white fluorescent light

In the second experiment, the effect of white fluorescent light (control) and white LED on the rooting and plantlets growth of 'Molly's White' and 'Anna's Red' was studied. The root number of both genotypes increased under the white LED compared to white fluorescent light (control) but only for 'Anna's Red' the difference was significant. The plantlets of 'Anna's Red' grown under white LED exhibited a higher root system quality than those under white fluorescent light. Compared to white fluorescent light (control), white LED did not influence the fresh weight and number of leaves of both genotypes during the rooting stage (Table 7).

The aim of the third experiment was to study the growth characteristics of the 'Molly's White' and 'Anna's Red' plantlets rooted *in vitro* in plastic vessels *versus* glass jars (control). The type of culture container had a significant impact on the root number of both genotypes. The higher number of roots was found in plastic vessels (5.73 and 5.08), appropriately for 'Anna's Red' and 'Molly's White', respectively. Only for 'Molly's White', the higher quality of root system was observed in plastic vessels (4.18), compared to glass jars (1.10). The fresh weight of 'Anna's Red' plantlets was significantly higher in plastic vessels than in glass jars (Table 8).

Table 7. Effect of white LED and white fluorescent light (control) on the fresh weight of plantlets, the number of roots, the quality assessment of root system and the number of leaves of *Helleborus* 'Molly's White' and 'Anna's Red' grown on the rooting medium (1 RM) at 20°C

'Molly's White'				
Color and source of light	Plantlets fresh weight (g)	Number of roots	The quality assessment of root system	Number of leaves
white fluorescent	0.50	5.08	4.18	3.60
white LED	0.61	7.65	5.00	4.25
	LSD 0.2062	*LSD 2.9887*	*LSD 1.2485*	*LSD 1.9065*
	SE 0.2014	*SE 0.7566*	*SE 0.3161*	*SE 0.4827*
'Anna's Red'				
Color and source of light	Plantlets fresh weight (g)	Number of roots	The quality assessment of root system	Number of leaves
white fluorescent	0.75	2.93	1.75	5.18
white LED	0.75	5.73*	4.43*	6.00
	LSD 0.2280	*LSD 2.2758*	*LSD 0.8816*	*LSD 1.2085*
	SE 0.0579	*SE 0.5761*	*SE 0.2232*	*SE 0.3060*

The quality assessment of root system (scale 0–5: 0 – no roots, 1 – very small roots, 2 – small roots, 3 – medium roots, 4 – large roots, 5 – very large roots
The assessment of the significance of differences was performed separately for each cultivar and analyzed feature,
LSD – Tukey's Least Significant Difference, SE – standard error, * Significant

Table 8. Effect of plastic vessels and glass jars (control) on the fresh weight of plantlets, the number of roots, the quality assessment of root system and the shoot length of *Helleborus* 'Anna's Red' and 'Molly's White' grown on the rooting medium (1 RM) at 20°C under white fluorescent light

'Molly's White'				
Type of vessels	Plantlets fresh weight (g)	Number of roots	The quality assessment of root system	Shoot length (mm)
plastic vessels	0.50	5.08*	4.18*	65.00
glass jars	0.51	1.43	1.10	65.00
	LSD 0.2623	*LSD 2.3626*	*LSD 1.7550*	*LSD 18.8066*
	SE 0.0758	*SE 0.6828*	*SE 0.5072*	*SE 5.4354*
'Anna's Red'				
Type of vessels	Plantlets fresh weight (g)	Number of roots	The quality assessment of root system	Shoot length (mm)
plastic vessels	0.75*	5.73*	1.75	64.58
glass jars	0.47	2.93	1.25	56.93
	LSD 0.2475	*LSD 2.2809*	*LSD 1.2946*	*LSD 32.4299*
	SE 0.0715	*SE 0.6592*	*SE 0.3742*	*SE 9.3728*

The quality assessment of root system (scale 0–5: 0 – no roots, 1 – very small roots, 2 – small roots, 3 – medium roots, 4 – large roots, 5 – very large roots
The assessment of the significance of differences was performed separately for each cultivar and analyzed feature,
LSD – Tukey's Least Significant Difference, SE – standard error, * Significant

In the fourth experiment, the post-effect of white, blue, and red light (LED) used during the storage of shoots at 15°C for 3 months on the subsequent *in vitro* rooting of 'Molly's White' and 'Anna's Red' was investigated. During storage, multiplied shoots were maintained in two different types of containers, plastic vessels and glass jars, on the 1 MM. All the shoots survived the 3-month storage in perfect conditions, regardless of the LED type (Fig. 3). After storage, shoots were transferred to the 1 MM and placed for 5 weeks at 20ºC under the white LED. Subsequently, the multiplied shoots were divided and placed on the 1 RM in plastic vessels and glass jars. Containers and LED used for storage and rooting did not clearly affect the percentage of rooted shoots of hellebore genotypes. The higher quality root system (4.65) was developed in 'Molly's White' grown in plastic vessels under red LED light. Only this result was statistically significant compared to the control (glass jars). On the other hand, the root development on the 'Anna's Red' shoots was very weak both in plastic vessels (1.6–2.1) and glass jars (1.9–2.2). For both cultivars the percentage of rooted shoots was from 80% to 100% (Table 9, Fig. 4).

***Ex vitro* acclimatization**

The post-effect of white, blue, and red light (LED) used during the storage of shoots on the survival and acclimatization of 'Molly's White' and 'Anna's Red' was observed. Well-rooted plantlets were planted in the greenhouse. It was observed that higher survival rates of 'Molly's White' were seen in a group of plants stored in plastic vessels (100%) compared to those in glass jars (87–88%). The quality assessment of root system of 'Molly's White' was similar in all treatments (from 4.0 to 4.9), regardless of the type of container and light (Figs. 5, 6). The percentage of 'Anna's Red' acclimatized plantlets was very high (100%) after storage in the white and blue LED but the quality assessment of root system was within the range 3.8–4.7. After storage in the red LED, a lower survival rate (75%) it was observed.

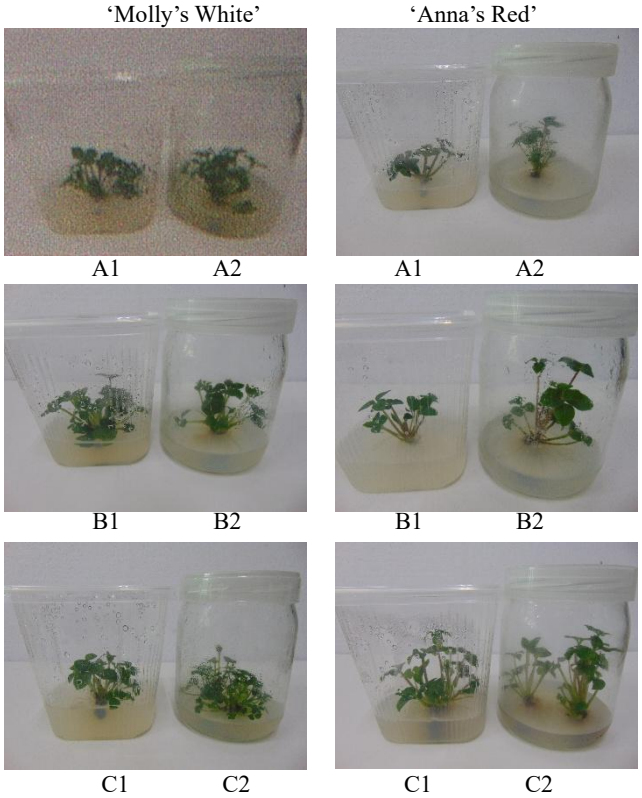

Figure 3. The effect of the LED (A – white, B – blue, C – red) and container type (1 – plastic vessel, 2 – glass jar) on the shoot survival and quality of hellebore 'Molly's White' and 'Anna's Red' stored at 15°C (3 months) on the multiplication medium (1 MM)

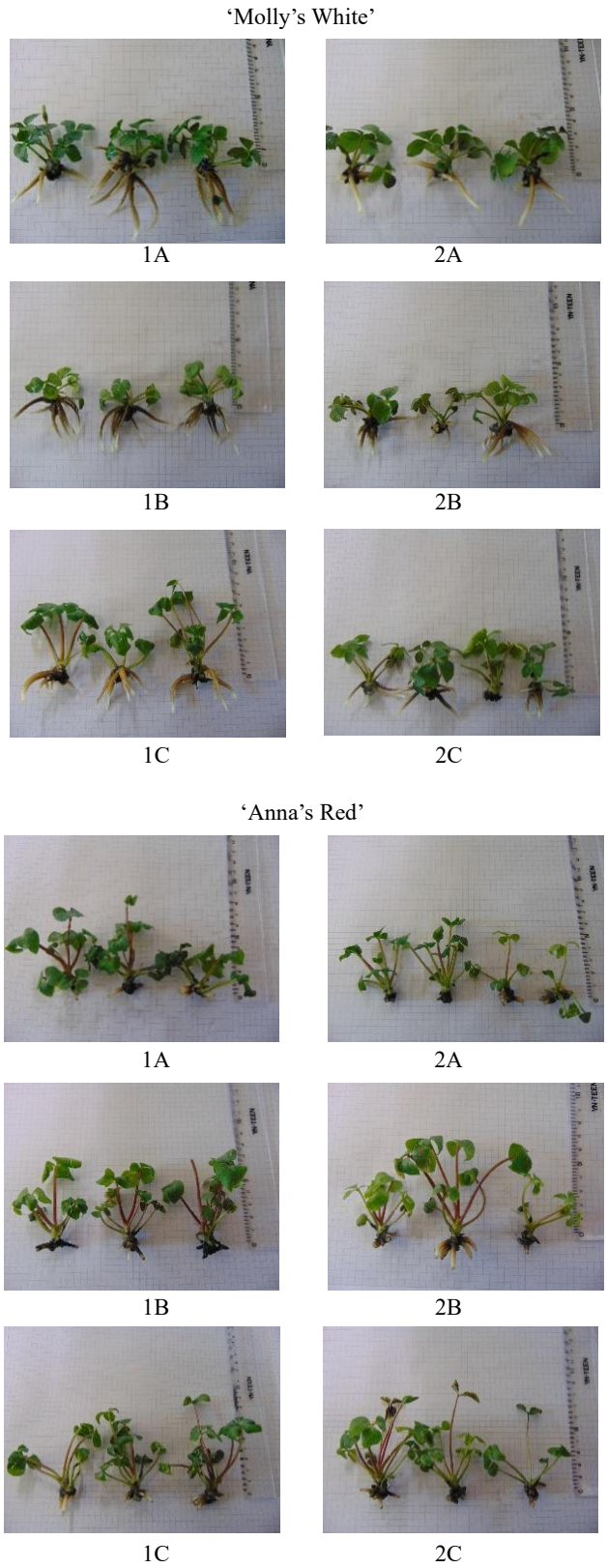

Figure 4. The post-effect of *in vitro* storage at 15°C for 3 months in the plastic vessels (1) and glass jars (2) and under different light (A – white, B – blue, C – red LED) on shoot rooting of *Helleborus* ‘Molly’s White’ and ‘Anna’s Red’ and then grown on the rooting medium (1 RM) at 20°C, under white LED

Table 9. The post-effect of *in vitro* storage on the quality assessment of root system of *Helleborus* 'Molly's White' and 'Anna's Red' stored in the plastic vessels and glass jars (control) at 15°C (3 months) under LED (white, blue, red) and then grown on the rooting medium (1 RM) at 20°C, under white LED

'Molly's White'				
Color of LED	Type of vessels	Percentage of rooted shoots (%)	The quality assessment of root system	
White	Glass jars	80	3.68	*LSD 1.7628*
	Plastic vessels	100	4.08	*SE 0.5095*
Blue	Glass jars	92	2.23	*LSD 2.1323*
	Plastic vessels	89	4.35	*SE 0.6163*
Red	Glass jars	100	2.58	*LSD 1.1289*
	Plastic vessels	100	4.65*	*SE 0.3263*
'Anna's Red'				
Color of LED	Type of vessels	Percentage of rooted shoots (%)	The quality assessment of root system	
White	Glass jars	90	1.85	*LSD 0.4577*
	Plastic vessels	86	1.60	*SE 0.1323*
Blue	Glass jars	100	2.18	*LSD 0.7254*
	Plastic vessels	89	1.50	*SE 0.2097*
Red	Glass jars	80	1.90	*LSD 0.8864*
	Plastic vessels	89	2.08	*SE 0.2562*

The quality assessment of root system (scale 0–5: 0 – no roots, 1 – very small roots, 2 – small roots, 3 – medium roots, 4 – large roots, 5 – very large roots
The assessment of the significance of differences was performed separately for each cultivar and type of light
LSD – Tukey's Least Significant Difference, SE – standard error, * Significant

Figure 5. The post-effect of *in vitro* storage on plantlets acclimatization of *Helleborus* 'Molly's White' and 'Anna's Red' stored in the plastic vessels (1) and glass jars (2) at 15°C (3 months) under white LED and subsequently rooted *in vitro* and acclimatized in the greenhouse

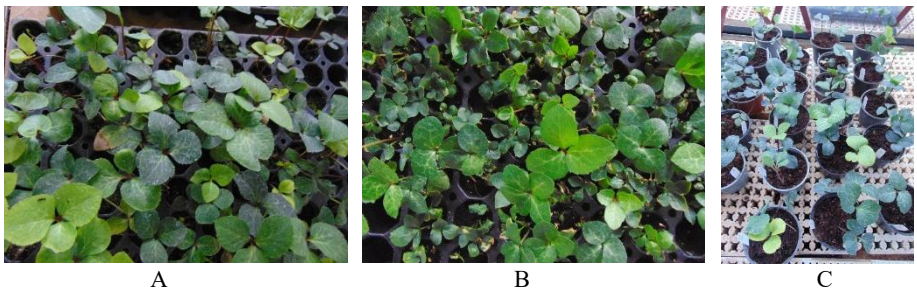

Figure 6. Plants of *Helleborus* 'Molly's White' (A) and 'Anna's Red' (B) 4 weeks after *ex vitro* acclimatization in the greenhouse (C)

DISCUSSION

Although tissue culture methods have been applied to micropropagate *Helleborus* species, the multiplication and rooting rates, as well as the acclimatization ability of these plants are currently limited in commercial *in vitro* production. This study presents optimization of the protocol for the *in vitro* propagation of the *Helleborus* 'Molly's White' and 'Anna's Red'.

The sucrose and mineral salts (mainly nitrogen salts) are more critical for axillary shoot branching and in maximizing the multiplication rate of both genotypes than the concentration of growth regulators in the multiplication medium. Lowering the sugar level to 20 $g \cdot L^{-1}$ in the multiplication medium containing 100% of MS mineral salts caused an increase in the value of the multiplication ratio by 119% for 'Molly's White' (from 1.13 to 2.47) and 50% for 'Anna's Red' (from 1.27 to 1.90) compared to the control. The use of this medium in commercial production will increase the efficiency of hellebore propagation. Few studies have presented the effects of sucrose and nitrogen salts on axillary bud activity of *in vitro* propagated *Helleborus* species, clones, or cultivars. For *H. purpurascens*, the highest multiplication rate of axillary shoots (2.3) was found on the medium supplemented with 30 $g \cdot L^{-1}$ sucrose, nitrogen salts of 50% of KNO_3 and 50% of NH_4NO_3, and a mixture of growth regulators: 2iP, BAP, kinetin, GA_3 (Gabryszewska 2014). In *H. niger*, moderate levels of sucrose (20–30 $g \cdot L^{-1}$) and half-strength of nitrogen salts (50% of KNO_3 and 50% of NH_4NO_3) in the media were beneficial for the development of axillary shoots and increased multiplication rate (Gabryszewska 2015). Also, Caesar and Adelberg (2015) reported that sucrose, nitrate, and the sucrose × phosphate interaction had the most significant positive effects on *Helleborus* × *nigercors* and *Helleborus* × *ballardiae* 'Raulston Remembered' multiplication ratio in liquid media. 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 30 $g \cdot L^{-1}$ sucrose and 6.25 mM phosphorus, and moderate levels of 40–50 mM nitrate. A high phosphate supply (6.25 mM) in the medium combined with a low level of sucrose (10 $g \cdot L^{-1}$) strongly inhibited the multiplication rate (Caesar 2015, Caesar & Adelberg 2015). In the present study, the high concentration of sucrose (50 $g \cdot L^{-1}$) and very high level of nitrogen salts (150% according to MS medium) also positively affect the multiplication rate (1.83) of 'Anna's Red'. This result suggests that the relation between sugar/nitrogen salts is more critical for axillary shoot branching than the absolute content of these substances in the medium. It was reported that the very high concentration of sucrose (80 $g \cdot L^{-1}$) strongly inhibited the growth of *H. purpurascens* and *H. niger* axillary shoots and simultaneously stimulated starch accumulation and induced the dormancy of axillary buds (Gabryszewska & Węgrzynowicz-Lesiak 2015).

In the present study, white LED significantly increased the multiplication rate, number, and fresh weight of shoots (especially during a passage extended to 5 weeks) of 'Molly's White' (2.96) and 'Anna's Red' (2.68) compared to white fluorescent light. These results confirm the findings of Nowakowska et al. (2023), who reported that white LED provided the highest multiplication rate and energy efficiency of *Helleborus* 'Molly's White' compared to fluorescent light, as well as improved morphology of shoots and biochemical profiles. Dhooghe and Van Labeke (2012) stated that the light quality (red, blue, red + blue, and white LED and white TL) used during *in vitro* propagation did not affect the multiplication rate of *H. orientalis* but influenced plant morphology. Caesar (2015) obtained a similar result, finding that the quality of LED light (blue and red wavelength) did not have a significant effect on the multiplication rate of *H.* × *hybridus*, *H.* × *nigercors*, and *H.* × *ballardiae* cultured *in vitro*.

The present study showed genotype-dependent multiplication rate of hellebore cultivars. The multiplication rate of 'Molly's White' genotype was a higher value than 'Anna's Red' on the optimal multiplication media (2 MM, 7 MM, and 8 MM). It has been previously reported that the multiplication rates of some *Helleborus* species (*H. argutifolius*, *H. foetidus*, *H. niger*, *H. purpurascens*, *H. orientalis*) and selected clones of *H. niger* (Seyring 2002; Poupet et al. 2006; Dhooghe & Van Labeke 2007; Gabryszewska 2014, 2015; Caesar 2015) are genotype-dependent. The multiplication rate was significantly higher for *H. niger* than for *H. argutifolius*, *H. foetidus*, *H. orientalis* (Dhooghe & Van Labeke 2007), and *H. purpurascens* (Gabryszewska 2014, 2015).

It has been reported that the *in vitro* rooting and the *ex vitro* acclimatization of hellebore plantlets are influenced by several factors, such as: genotype,

growth regulators, environmental factors (sucrose/nitrogen salts ratio in the medium, temperature, LED) and inoculation plants with bacteria *Burkholderia phytofirmans* (Seyring 2002; Poupet et al. 2006; Dhooghe & Van Labeke 2007, 2012; Beruto & Curir 2009; Beruto et al. 2013; Orlikowska et al. 2013; Gabryszewska 2014, 2015, 2017; Caesar 2015; Matysiak & Gabryszewska 2016).

In the present study, we tested the post-effect of various multiplication media (1 MM–8 MM) and three media for rooting (1 RM–3 RM) for *Helleborus* 'Molly's White' and 'Anna's Red'. It was stated that the composition of the medium used in the multiplication stage markedly affected the efficiency of subsequent rooting of both genotypes. It was interesting that shoots of both genotypes grown on the best multiplication media (2 MM, 3 MM, 7 MM, 8 MM) show the highest rooting capacity and the best developed root system. The presented results indicate that the sucrose/nitrogen ratio in the multiplication medium has more critical effect on subsequent rooting of both genotypes than type and concentration of growth regulators and composition of rooting media. In the case of *H. purpurascens* and *H. niger,* it has been found that root regeneration is strongly dependent on the sucrose or sucrose/nitrogen salt relationship in the MS rooting media (Gabryszewska 2014, 2015). Sucrose (30 and 50 $g \cdot L^{-1}$) in the presence of nitrogen salts in the half concentration (50% according to MS medium) strongly increased the number of roots and percentage of rooted shoots in *H. purpurascens* compared to lower (10 $g \cdot L^{-1}$) and higher levels of sucrose in the rooting medium (Gabryszewska 2014). In the case of *H. niger*, sucrose at concentrations of 50 $g \cdot L^{-1}$ strongly stimulated root formation on the media with a reduced level of nitrogen salts (25% and 50%). An increase in the level of nitrogen salts in the medium (100%) markedly inhibited root formation (Gabryszewska 2015). Beruto and Curir (2009) demonstrated that the type of cytokinin used during the multiplication stage can markedly affect the efficiency of subsequent rooting of *H. niger* and several *H.* × *nigercors* genotypes. The use of kinetin in the multiplication medium enhances subsequent rooting, while BAP at high concentration decreases the rooting percentage. Hellebore shoots multiplied on MS macroelements showed better rooting efficiency compared to those propagated on Quorin and Lepoivre macroelements (Beruto et al. 2013).

It was observed that container effects on rooting depended on the specific growth parameters being studied. The plastic vessels had a significant and positive influence on the number of roots on both genotypes. In the glass jars, only a few roots were formed. Despite inconsistent research results, plastic vessels appeared to be more effective for *in vitro* rooting and subsequent transport of plantlets. Different types of containers were used at all stages of *in vitro* propagation. The most essential features required of vessels are to provide homogeneous and sufficient light quality, prevent microorganism contamination, and allow gas exchange (Naik et al. 2020).

Light is an essential factor for the growth and development of plantlets *in vitro*. LEDs have demonstrated promising results in plant tissue culture applications (Gupta & Jatothu 2013). The white LEDs have been shown to significantly increase the number and quality of roots in 'Anna's Red' compared to white fluorescent light. This effect was weaker and not significant in the case of 'Molly's White' genotype. It was found that red LED stimulated the number of roots on *H. orientalis* microshoots. Red and white LED, as well as TL (control), promoted the formation of longer roots than blue and red + blue LED (Dhooghe & Van Labeke 2012). The LEDs had no significant effect on fresh weight of microplants or leaf number in both genotypes. Similar results were obtained for *in vitro* rooting of Marubakaido apple tree rootstocks (Tomazini Scolaro et al. 2025). The number of roots obtained using LED treatments was equivalent to that obtained using the control (fluorescent light).

An innovative study evaluated the effect of different light spectra on the conservation of the banana 'Prata Catarina' under slow-grown *in vitro* storage (Rodrigues et al. 2022). Plantlets grown under the blue spectrum showed a positive response to slow-grown storage. Our study demonstrated that storing hellebore shoots *in vitro* under white, blue, and red LED (at 15°C for 3 months) did not negatively affect subsequent growth, shoot rooting ability, and the acclimatization rate of microplants. After storage, the quality of shoots was excellent – no shoot necrosis and yellow or dead leaves. Then the shoots of both genotypes were multiplied in a single passage and rooted *in vitro* in plastic vessels and glass jars under white LED. The type of LED used during storage does not significantly affect the rooting ability and development of roots of 'Molly's White' and 'Anna's Red'. It was observed that

a high rooting rate and a well-developed root system were achieved after storing shoots under all LED types. Storage of 'Molly's White' under red light resulted in 100% rooted shoots in both container types, but the best root system development was only in plastic vessels. The post-effect of white, blue, and red light (LED) used during the storage of hellebore cultures was weaker on *ex vitro* acclimatization than on *in vitro* rooting. Plantlets stored under red LEDs exhibited lower acclimatization rates in both types of containers compared to those stored under white or blue LED.

The type of containers (different shapes, materials, volume, and gas permeability) used to maximize the storage duration of shoot cultures is an essential factor. The following containers for slow-grown *in vitro* storage were tested: traditional glass jars or tubes, glass jars with ventilated polyethylene caps, Starpac Pac ™, HEPA-filter-equipped culture vessels, GA7® magentas (Lisek & Orlikowska 2008; Benelli et al. 2022; Vollmer et al. 2024). Trejgell et al. (2015) demonstrated that polycarbonate boxes were more suitable for storing *Senecio macrophyllus* shoots than glass vessels. In addition to the containers, several factors can influence the quality of shoots during *in vitro* storage, including temperature, the presence or absence of light and its intensity (Benelli et al. 2022). White fluorescent light used during storage negatively affected the culture viability, multiplication rate, and rooting response of *S. macrophyllus* (Trejgell et al. 2015).

Another critical factor during storage is temperature. Incubation at a temperature below the optimal growth temperature will reduce the metabolic activities. Cold storage is often combined with reduced light intensity or total darkness. However, different combinations of photoperiod and light intensity during storage were more effective than total darkness (Benelli et al. 2022). A chilling treatment (5°C) of *H.* × *nigercors* shoots enhanced rooting rate by 5% and the rooting rate reached approximately 80% (Beruto & Curir 2009, Beruto et al. 2013). Rooted shoots of *H. purpurascens* survived and acclimated in the greenhouse after chilling at 5°C for 2 months in total darkness. Plantlets that were not cooled died (Gabryszewska 2014). A low temperature (15°C) and a sucrose concentration of 30–50 $g \cdot L^{-1}$, combined with rooting medium with reduced nitrogen salts to 50% of MS, were optimal for *ex vitro* acclimatization of *H. niger* plantlets (Matysiak & Gabryszewska 2016).

Presented results of industrial and development research were the basis for developing an optimal procedure for propagating *Helleborus* 'Molly's White' and 'Anna's Red' *in vitro*.

CONCLUSIONS

The results presented here show that sucrose and mineral salts (especially nitrogen salts) appeared to be more critical for axillary shoot branching (multiplication rate) and subsequent *in vitro* rooting for both genotypes of hellebore than the concentration of growth regulators present in the multiplication and rooting media. The composition of the medium used for the multiplication markedly affected the efficiency of subsequent rooting of both genotypes. The sucrose/mineral salts ratio in the multiplication medium has a greater effect on subsequent rooting of both genotypes than the type and concentration of growth regulators and composition of the rooting media.

The white LED increased the multiplication and rooting rates of both genotypes compared with white fluorescent light. Plastic vessels have a significant and positive effect on root number in both genotypes. The post-effect of white, blue, and red light (LED) used during the storage of cultures of hellebore genotypes was weaker on the *ex vitro* acclimatization than on the *in vitro* rooting.

The genotype-dependence of multiplication and *in vitro* rooting, and *ex vitro* acclimatization of *Helleborus* 'Molly's White' and 'Anna's Red' was stated.

The optimal composition of the multiplication and rooting medium, LED, and plastic culture vessels will significantly increase the efficiency of *in vitro* propagation of hellebores for commercial production.

Author contribution statement

EAG – A – Research concept and design, C – Data analysis and interpretation, D – Writing the article
KN – A – Research concept and design
PN – A – Research concept and design
DM – B – Collection and/or assembly of data

Conflict of interest

The authors declare that there is no conflict of interest in this report.

Funding source
This research is a part of the project: "Development of effective and mass micropropagation and acclimatization technology of potato, cardoon, rhubarb, purple coneflower and hellebore by the use of different types of LED light and media composition to obtain high quality plant material for culture". This project was partially funded by NCRD – Ścieżka dla Mazowsza/2019.

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