In vitro morphogenesis, cryopreservation and induction of variability in bleeding heart (*Lamprocapnos spectabilis* (L.) Fukuhara): A review

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Abstract

This review explores recent advances in the biotechnology of *Lamprocapnos spectabilis* (L.) Fukuhara (commonly known as bleeding heart), a valuable ornamental-medicinal perennial. The article covers *in vitro* morphogenesis, cryopreservation techniques, and methods for inducing variability. The establishment of *in vitro* cultures utilized Murashige and Skoog medium enriched with various auxins, cytokinins, gold nanoparticles, and plant extracts, under both fluorescent and wide-spectrum LED lighting. Axillary bud activation and indirect somatic embryogenesis were more efficient, particularly in the presence of kinetin and picloram, respectively, compared to adventitious shoot regeneration. Significant cultivar differences were observed, with 'Valentine' being the easiest and 'White Gold' the most challenging to culture *in vitro*. To mitigate stress caused by classical growth regulators, alternative substances such as nanoparticles and natural extracts were used. Gold nanoparticles enhanced shoot proliferation and plantlet quality, while coconut and rice extracts improved survival rates during acclimatization. Enhanced metabolite production was achieved using exogenous auxins and gold nanoparticles. Guaiacol peroxidase was identified as a sensitive oxidative stress marker, with glutathione reductase being the most stable under stress. Cryogenic techniques incorporating explant encapsulation, i.e. encapsulation-vitrification, showed high effectiveness and genetic stability of plants, with nanomaterials boosting effectiveness. Coconut extract also enhanced *post*-thaw shoot proliferation, while sesame extract served as a natural retardant for slow-growth cultures. Mutagenic effectiveness ranked as microwaves < nanoparticles < X-rays. Comprehensive genetic variability insights were provided by integrating multiple SPAR marker systems. This review underscores the promising biotechnological advancements for *L. spectabilis*, emphasizing the potential of *in vitro* techniques, innovative cryopreservation methods, and the application of nanoparticles and plant extracts to enhance micropropagation, genetic variability, and metabolite

production, thereby contributing to the conservation and commercial sustainability of this valuable ornamental-medicinal perennial.

Keywords: metabolism, nanoparticles, plant extracts, ornamental plants, stress reaction, tissue culture

Introduction

Lamprocapnos spectabilis (L.) Fukuhara is a herbaceous perennial native to Siberia, northern China, Korea, and Japan, where it has been known for at least 2000 years (Hodges 2012; Kim et al. 2018). Due to its unique flower shape, arranged in unilaterally pendulous racemes or spikes, this species is also known by the common names bleeding heart, lady in a bath, and lyre flower. In English literature, other terms such as fallopian buds and lady's locket also appear. According to various taxonomies, this species is placed in a separate, small botanical family Fumariaceae or the subfamily Fumarioideae belonging to Papaveraceae (Kamińska et al. 2005; Frey and Moretti 2019). In 1997, bleeding heart was reclassified (based on the morphology, as well as the data of nuclear ribosomal DNA internal transcribed spacer (nrDNA-ITS) and rpsl6 intron sequences) to the monotypic genus *Lamprocapnos* (Lidén et al. 1997). Nevertheless, it is often referred to in literature and horticultural practice under its previous name *Dicentra spectabilis* (L.) Lem. (syn. *Fumaria spectabilis* L.) (Cho 2018; Igori et al. 2023). This cold-hardy species occurs in temperate climates, although it can also be found in south-central Alaskan home gardens (Robertson 2013). It is a long-day plant, flowering in late spring (April to June) - at the peak of the floristic season (Roberts et al. 1995). The seeds of bleeding heart are black-colored and of spherical shape with white large elaiosome to be dispersed by ants (Kim et al. 2011). Mature plants produce fleshy tuberous roots (Kamińska et al. 2005). Due to its decorative leaves (varying shades of green or gold-yellow depending on the cultivar) and spectacular white, pink, or red flowers (Fig. 1), bleeding heart has been used in Europe and America for landscape architecture since the 19th century in plantings in parks, gardens, balconies, as a houseplant, and in floristry as a cut flower. The flowers are bisymmetric (as opposed to the actinomorphic flowers found in other Papaveraceae species), measuring 20–25 mm in width. They are flattened with a heart-shaped base and have two lanceolate, deciduous sepals that are 3–4 mm in length (Zhang and Zhao 2018). Currently, methods for controlled cultivation of bleeding heart are known, allowing for year-round production *in vivo* (Hodges 2012). Due to the long vase life of cut flowers (lasting

from 8 to even 17 days), this species is particularly popular for Valentine's Day and Mother's Day (Roberts et al. 1995).

L. spectabilis may also find applications in medicine, pharmacology, and the cosmetic industry due to its high content of health-promoting isoquinoline alkaloids: protopine and sanguinarine (Och et al. 2017; Hyeon Kim et al. 2018; Adamski et al. 2020). The roots of bleeding heart are used in Asian folk medicine for treating ulcers and paralysis (Iwasa and Kim 1997). It has been also applied for the treatment of strokes, bruises, and blood circulation (Kim et al. 2017). The extract obtained from *L. spectabilis*, when applied to the skin even at low concentrations (0.1%), slows down UV-induced aging (Lee et al. 2004a). This species is also a source of antifungal and antibacterial substances, effective for example in combating methicillin-resistant *Staphylococcus aureus* F.J. Rosenbach strains (MRSA) (Ma et al. 2000). Studies conducted by McNulty et al. (2007) and Petruczynik et al. (2019) demonstrated the presence of substances with antidepressant properties in bleeding heart extracts, as well as biologically active lactones effective in eliminating human cancer cell lines (squamous cell carcinoma and adenocarcinoma). Therefore, it is worth paying more attention to this species and introducing it to *in vitro* conditions.

Tissue cultures can be used in plants for the following purposes: reproduction (micropropagation), obtaining secondary metabolites, storage and protection of genetic resources, improvement, and breeding (inducing variability). This technology is commonly used in various ornamental and medicinal plants, including bleeding heart (Rout and Jain 2020). The objective of this review is to provide a comprehensive summary of the vegetative methods for *in vitro* propagation and long-term storage of *L. spectabilis* tissues in liquid nitrogen, while also exploring strategies to enhance genetic diversity within this species through both classical and modern biotechnological approaches.

Development of a model system for vegetative propagation of bleeding heart *in vitro*

Bleeding heart is mainly propagated vegetatively through cuttings and division of fleshy roots, or less commonly through seeds (self-pollination can occur) (Sim et al. 2005; Hodges 2012). Seed viability is low and they should be sown immediately after collection (Deno 1993). Subsequently, the seeds must undergo a winter dormancy period to germinate in the spring of the following year. Thus, the entire developmental cycle of the plant takes nearly a year (Cho et al. 2020). Moreover, the generative propagation method does not guarantee the maintenance of maternal plant characteristics. Cutting and division *in vivo* ensure obtaining true-to-type clones and earlier flowering, however, the efficiency of these methods is limited

and dependent on climatic conditions. Additionally, the content of valuable secondary metabolites in plants cultivated *in vivo* is usually low and subject to significant fluctuations (Mohammad et al. 2014). *In vitro* technologies may provide a solution to these problems.

Until 2019, information on tissue cultures of bleeding heart was limited to the induction of indirect somatic embryogenesis from seeds or callus suspension culture in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) (Lee and Lee 2003; Lee et al. 2004b). However, somatic embryogenesis is a complex process associated with specific difficulties, such as asynchronous embryo development, lack of conversion into complete plants, and/or disruption of genetic stability in plants regenerated via callus. In studies by Lee and Lee (2003) and Lee et al. (2004b), 64% conversion of somatic embryos was achieved, of which only 46% survived acclimatization to greenhouse conditions. Moreover, suspension cultures are susceptible to contamination, and seed availability in bleeding heart is limited. Meristematic explants (apical and lateral buds) or non-meristematic explants (internodes, leaves, and their fragments) are much easier to obtain and allow the use of other micropropagation techniques besides the aforementioned somatic embryogenesis, i.e., activation of lateral shoots and adventitious organogenesis (Fig. 2). These techniques allow for easy and quick production of a significant number of offspring plants. However, to fully utilize the potential of various micropropagation methods, it is necessary to conduct numerous experiments on different cultivars, considering factors such as type, age, and size of explant, type, and concentration of growth regulators, and their mutual interaction in the medium, as well as the influence of optical radiation (Moraes et al. 2021).

Effect of growth regulators on the regeneration of meristematic explants

Activation of axillary buds

The morphogenetic response of meristematic explants (nodal segments) in bleeding heart was dependent on the genetic factor. For example, the proliferation and development of axillary shoots in the 'Gold Heart' cultivar were most effectively stimulated by kinetin (KIN), while for the 'White Gold' cultivar, MS medium (Murashige and Skoog 1962) with simultaneous addition of indole-3-butyric acid (IBA) and 6-benzyladenine (BA) proved to be the most optimal in terms of multiplication rate (9.8) and biometric parameters of shoots (Kulus 2020a, Kulus et al. 2021). These results are consistent with reports by Zagorskaya and Yegorova (2018) on the varied micropropagation efficiency of different plant cultivars. The relatively high dry matter content confirmed the high quality of the obtained microshoots without signs of hyperhydration, encountered in commercial laboratories (Vitroflora Ltd, Poland, personal

communication). Nevertheless, an additional subculture of shoots onto a medium containing only auxin proved necessary to induce rhizogenesis. 1-Naphthaleneacetic acid (NAA) was most effective in initiating this process, while Indole-3-acetic acid (IAA) stimulated root elongation. Despite the need to separate the multiplication and rooting stages, it was possible to obtain complete bleeding heart plants *in vitro* in a relatively short time (Kulus 2020a, Kulus et al. 2021). On the other hand, it was found that thidiazuron (TDZ), NAA, and picloram (PIC) inhibit caulogenesis in *L. spectabilis*, inducing intensive callus development instead (Kulus 2020a), which is consistent with reports on other plant species (Simon and Petrášek 2011; Zhang et al. 2011; Kawochar et al. 2017).

Somatic embryogenesis

Indirect somatic embryogenesis was initiated after placing nodal explants on media containing PIC or NAA. According to Méndez-Hernández et al. (2019), auxins affect gene expression, including transcription factors (TF), which cause somatic cells to acquire embryogenic potential, which could explain the obtained results. In bleeding heart, somatic embryos were observed at all developmental stages described in scientific literature (Joshi and Kumar 2013). Additionally, groups of polyembryos and secondary adventitious embryos were formed. The highest number of somatic embryos per explant (12) was obtained on MS medium with 0.5 $mg \cdot L^{-1}$ PIC (Kulus 2020a). Considering that the explants used in the experiment were only 5-10 mm long, this method seems to be efficient for the micropropagation of bleeding heart or transgenesis research.

Acclimatization

Another crucial stage of micropropagation is plant acclimatization in the greenhouse (Fig. 2E). The survival rate of bleeding heart microshoots after transfer to *ex vitro* conditions varied greatly, depending on the composition of the medium used for shoot multiplication (Kulus et al. 2021). A positive correlation was observed between the number of leaves on the shoot and the viability of acclimatized plants, which may be related to increased activity of the photosynthetic apparatus. A significant influence of the shoot multiplication medium composition on the quality of greenhouse-grown plants was also found. The presence of IBA in the multiplication medium was favorable with bleeding heart ‘White Gold’ in contrast to IAA-supplemented media. This effect, however, was not observed with the cultivar ‘Gold Heart’. Genetic analyses of RAPD (Randomly Amplified Polymorphic DNA (Williams et al. 1990)) and SCoT (Start Codon Targeted Polymorphisms (Collard and Mackill 2009)) markers confirmed the stability of plants from experimental objects ensuring the highest multiplication

rate (Kulus et al. 2021). This fact is crucial for the possibility of using the developed protocols in commercial micropropagation of *L. spectabilis*.

Effect of growth regulators on the regeneration of non-meristematic explants

Due to the limited productivity of lateral meristems, Kulus and Tymoszuk (2020) focused on the possibility of using non-meristematic shoot fragments in *in vitro* systems of bleeding heart. Explants of the 'Alba' cultivar derived from whole leaves, petioles, and internodes were placed on a medium supplemented with BA, IAA, NAA, 2,4-D, or PIC in various concentrations and combinations.

Adventitious organogenesis

The efficiency of callogenesis was independent of the explant type. However, the influence of explant type and medium composition on the dry and fresh mass of the obtained callus was confirmed. The results were surprising, as despite using 17 combinations of growth regulators, only 2.5% of explants regenerated adventitious shoots. Rhizogenesis was observed in 4.5% of explants (Kulus and Tymoszuk 2020). The obtained efficiency of adventitious organogenesis is lower than that described in other botanical families (Tymoszuk and Miler 2019). This suggests that bleeding heart may be classified as a difficult species in micropropagation. Similar problems with stimulating *in vitro* regeneration have also been described in other members of the Papaveraceae (Park et al. 2004), which may be a characteristic feature of this family.

Somatic embryogenesis

The efficiency of somatic embryogenesis in *L. spectabilis* was significantly higher, although dependent on the medium composition and explant type (somatic embryos were formed by 0 to 100% of explants). Embryogenic callus was most often formed on leaves and least often on internodes (Fig. 2D). The highest number of somatic embryos per explant (11.4) was formed on petioles on medium with 0.5 $mg \cdot L^{-1}$ BA and 1.0 $mg \cdot L^{-1}$ PIC (Kulus and Tymoszuk 2020). The auxin 2,4-D was also effective in inducing somatic embryogenesis. Joshi and Kumar (2013) report that synthetic auxins often trigger the embryogenic potential of cells and stimulate embryo proliferation at an early developmental stage while inhibiting the expression of genes responsible for their further maturation. These reports were partially reflected in studies on bleeding heart, as the majority of somatic embryos were indeed at an early developmental stage; nevertheless, germinating embryos with clearly visible embryonic roots were also observed (Kulus and Tymoszuk 2020).

Multidirectional effects of plant extracts and nanoparticles (NPs) in micropropagation of bleeding heart

Bleeding heart plants produced on media supplemented with traditional growth regulators often had noticeably paler leaf color compared to the untreated controls (Kulus et al. 2021). A probable explanation for this phenomenon is the adverse physiological changes caused by cytokinin BA, also described by other authors (Bidabadi and Jain 2020; Manokari et al. 2021). It was also found that the simultaneous addition of auxins and cytokinins to the medium (even at low concentrations) resulted in abundant polyphenol secretion (Fig. 2B), which inhibited plant growth (Kulus et al. 2021). Therefore, efforts were made to use substitutes for synthetic growth regulators in *L. spectabilis in vitro* cultures, i.e., plant extracts and nanoparticles.

Plant extracts can be a cheaper and more natural source of phytohormones, vitamins, nutrients (including sugars), phenols, and proteins beneficial for plants (Gnasekaran et al. 2010, Markin et al. 2023). Some extracts may also contain natural growth retardants, which are an interesting alternative to osmotically active substances and synthetic compounds added to the medium for storing genetic resources under slow growth conditions. Over the years, extracts of various origins have been added to *in vitro* media mostly for orchid propagation (Molnár et al. 2011, Venkatachalam et al. 2015). In the experiment by Kulus and Miler (2021), the usefulness of traditional growth regulators and plant extracts obtained from coconut pulp and oat, rice, and sesame seeds in the activation and proliferation of lateral shoots of two *L. spectabilis* cultivars was compared. The latter three types of extracts were used for the first time in plant *in vitro* cultures. To learn more about their role in the morphogenetic response of explants, this study included an analysis of the chemical composition of the extracts and compared the costs of preparing the media (Kulus and Miler 2021). The effect of the applied extracts on micropropagation efficiency usually depended on the cultivar, but also on the studied plant trait. Coconut extract stimulated shoot proliferation and increased the multiplication rate in the 'Gold Heart' cultivar. Rice extract, on the other hand, stimulated callogenesis in bleeding heart 'White Gold' and provided a nearly twofold higher multiplication rate than conventional growth regulators and, importantly, slowed down the plant aging process (Kulus and Miler 2021). A similar plant response to the addition of natural supplements to the medium was observed in *Pogostemon cablin* Benth. (Swamy et al. 2014). An additional advantage of rice extract is its nearly four times lower cost of production compared to commercial auxins and cytokinins. This is of great importance, as the costs of purchasing reagents in laboratory plant production range from 20 to 40% of all financial outlays (Tomar et al. 2007; Chen 2016). Sesame extract, in turn, inhibited the development of

explants in both studied cultivars, probably due to the relatively high content of polyphenols (Kulus and Miler 2021). Therefore, it can be successfully used in the slow-growth culture of bleeding heart. An additional advantage of using natural plant products was the easier disposal of used medium, which after appropriate processing, can be used as fertilizer in further *ex vitro* cultivation.

A breakthrough in developing micropropagation protocols for *L. spectabilis* was the use of nanomaterials by Kulus et al. (2022). Nanoparticles are structures with dimensions from 1 to 100 nm. Compared to conventional materials, they are characterized by higher chemical reactivity and unique physical properties (Fayez et al. 2017). It is known that nanoparticles can interact directly with the cell, affecting plant growth and metabolic activity positively or negatively, depending on the parameters of the nanoparticles, i.e., their type, concentration, method of synthesis, diameter, shape, etc., as well as the properties of the plant material itself (genotype, organ, anatomical structure) (Sanzari et al. 2019). Currently, many types of nanoparticles are synthesized, among which silver nanoparticles (AgNPs) are most often used in plant research (Tymoszuk and Miler 2019). Overall, however, there are still many unknowns regarding the impact of nanoparticles on living organisms and the environment, especially on the possibility of using them in *in vitro* cultures. The addition of gold nanoparticles to the medium, regardless of their concentration (50 – 100 ppm), positively affected not only the proliferation of lateral shoots of bleeding heart ‘Valentine’ but also their further growth *in vitro* (Fig. 2C) compared with the plane MS medium (Fig. 2A). The multiplication rate in the presence of NPs reached a value close to 23, more than twice as high as in the case of classical growth regulators. No effect of nanoparticles on the relative water content in shoots or the efficiency of rhizogenesis in the rooting medium (100%) was found. Nanoparticles, however, stimulated elongation and "branching" of roots, which emphasizes their usefulness (Kulus et al. 2022).

Effect of wide-spectrum light-emitting diodes on the *in vitro* morphogenesis of bleeding heart

Light is a crucial factor influencing plant growth and development (Paik and Huq 2019). Currently, increasing attention is being paid to the possibility of using modern light sources such as LEDs (Light Emitting Diodes) in micropropagation. Apart from economic and environmental aspects (longer diode lifespan, lower power consumption, and less heat emission compared to traditional fluorescent lamps), the advantage of using LEDs in plant cultivation is the ability to precisely control the optical spectrum. Previous plant studies have

mainly focused on red and blue light (Bello-Bello et al. 2017). However, the latest research indicates that plants require a richer spectrum for full functional efficiency.

In the article by Miler et al. (2019), the impact of optical spectrum composition on the effectiveness and economy of micropropagation of five ornamental plant species, including bleeding heart, was verified. For this purpose, wide-spectrum LEDs and standard fluorescent lamps were used. The results indicated that the selection of light conditions in the growth room/phytotron should be adapted to the individual needs of particular plant species, which is consistent with reports by Gupta and Jatothu (2013). LED modules with high content of red and far-red light, crucial for photosynthesis efficiency, are optimal for the *in vitro* growth and micropropagation of bleeding heart. Such a spectrum allows for obtaining a multiplication rate and plant biometric parameters comparable to those observed under fluorescent lamps, with simultaneously twice lower electricity consumption. Lamps with increased blue and green light content do cause a significant reduction in bleeding heart shoot length, but the electricity costs necessary to produce 1 million plants using these modules are also four times lower (Miler et al. 2019). This is particularly important from the producers' point of view, as electricity costs are among the highest in commercial *in vitro* laboratories (Chen 2016) and can constitute from 20% to even 60% of all micropropagation costs, depending on the geographical location of the laboratory (Tomar et al. 2007). Regardless of the light source used, it was not possible to induce simultaneous rooting of plants at the multiplication phase. For comparison, the closely related *Dicentra* × *hybrida* regenerates complete shoots and roots on MS medium without the addition of growth regulators and under classic fluorescent lamps, with 100% acclimatization success (Kulus 2021). This emphasizes the difficulty of cultivating *L. spectabilis* under *in vitro* conditions.

Development of cryopreservation protocols and their impact on the stability of *L. spectabilis*

Biodiversity conservation is one of the most important challenges of the modern world. Although commercial cultivars of bleeding heart are popular in cultivation, the number of endemic populations of this species is small, and it may be classified as endangered in the future (Hammer et al. 2005). For short-term storage, propagation, and transport, artificial seeds can be useful (Gantait et al. 2015). However, cryopreservation, which involves storing tissues at the cryogenic temperature of liquid nitrogen (-196°C), is considered the most effective method for long-term biodiversity conservation (El Merzougui et al. 2023; Sochacki et al. 2024). This approach can also be useful for storing cell lines with a particularly high

capacity for producing valuable secondary metabolites. In the available literature before 2019, there was no information on the use of artificial seeds and cryogenic techniques for *L. spectabilis*. Maintaining viable biological material in liquid nitrogen is possible, provided that the tissues are properly prepared (El Merzougui et al. 2023). Cryopreservation is a complex process that requires careful optimization of several key stages. The choice of cryopreservation method (vitrification, droplet-vitrification, encapsulation-vitrification, or encapsulation-dehydration) depends on the species. It is then necessary to optimize the composition of the pre-culture medium, the first stage of the procedure, which aims to pre-harden the cells. In the next stage, it is crucial to select the appropriate plant vitrification solution (PVS), a mixture of dehydrating, protective, and stabilizing substances (cryoprotectants). The concentration of PVS and the treatment time must be considered. In the encapsulation-dehydration technique, it is necessary to optimize the drying time. The prepared plant material is placed in liquid nitrogen, but to regenerate a complete plant, the thawed explants must be placed on a growth medium with an appropriately selected composition (Popova et al. 2023).

Kulus (2020b) aimed to develop a simple cryopreservation procedure based on drying explants with sterile air, without the need for chemical cryoprotectants. However, it was found that the apical buds of *L. spectabilis* have limited tolerance to prolonged desiccation. Despite considering several experimental factors, the effectiveness of this technique was limited (survival rate of 31.3 – 36.4%). Interestingly, the explants that survived freezing produced longer shoots with significantly greater mass than untreated controls. The improvement in plant quality was also evident during the planimetric analysis of leaf size (Kulus 2020b). A similar positive effect of stress on plant development was described by Adamczuk et al. (2012) in the *in vitro* cultures of flax and oat. The observed beneficial effect of encapsulation on the growth and development of non-frozen explants highlights the relevance of using artificial seeds for storage, transport, and propagation of bleeding heart.

Given the limited effectiveness of the encapsulation-dehydration technique, three other cryopreservation methods were used: vitrification, droplet-vitrification, and encapsulation-vitrification (Kulus 2020c, d). The regenerated plant material was then subjected to detailed, multi-level analysis using various analytical methods. Comparative analyses indicated that vitrification solution 3 (PVS3) (Nishizawa et al. 1993) was the most effective in securing cryo-treated tissues of *L. spectabilis*, both in terms of explant survival and the quality of the plants obtained from them (Kulus 2020d). Furthermore, numerous experiments revealed that the tolerance of encapsulated bleeding heart explants to prolonged dehydration is significantly

higher than that of non-encapsulated apical buds, regardless of storage in liquid nitrogen (Kulus 2020c). The relevance of supplementing the alginate capsule with salts and vitamins from the MS medium and the impact of this treatment on internode elongation and leaf development was also highlighted (Kulus 2020d). The encapsulation-vitrification technique, based on shoot tip (2-4 mm) preculture on the MS medium with 1.0 $mg \cdot L^{-1}$ KIN, 9% sucrose, and 10 μM abscisic acid (ABA), encapsulation in 3% calcium alginate, followed by 20 min exposure to loading solution (LS, 2.0 M glycerol and 0.4 M sucrose) and 150-min dehydration with PVS3, ensured the highest survival rate of biological material (over 73%), as well as the most intensive plant development after thawing explants, including shoot proliferation and root regeneration, which was not observed in the control object. The lowest cryopreservation efficiency was obtained with the vitrification technique (Kulus 2020c). Interestingly, the opposite results were reported in *Chrysanthemum* × *morifolium* /Ramat./ Hemsl. 'Escort' (Halmagyi et al. 2004). Another advantage of the developed procedure was the stable viability of explants, which did not change in time. All viable apical buds resumed growth, which is not always evident in post-freezing plant culture (Osorio-Saenz et al. 2011).

Spectral analyses conducted in the next stage of the research helped to understand the impact of the cryopreservation procedure on the biochemical activity of plants (Kulus 2020c; Kulus and Tymoszuk 2021). The chlorophyll content was significantly lower in shoots obtained from cryopreserved meristems compared to the control, which is likely due to the high lability of these pigments described by Van Assche and Clijsters (1990). It was also found that in response to moderately strong stress, *L. spectabilis* plants exhibit increased anthocyanin production, which significantly decreases with prolonged exposure to the stressor (Kulus 2020c).

The ultimate indicator of the effectiveness of the cryopreservation procedure is the absence of genetic changes in the stored biological material (Adhikari et al. 2020). ISSR (Inter Sequence Simple Repeat (Zietkiewicz et al. 1994)), RAPD, and SCoT molecular analyses, optimized for this species by Kulus (2020b, c), demonstrated the effectiveness of encapsulation-based methods in securing genetic stability (100% DNA sequence homology with untreated control). Slight genetic variation was detected by RAPD and ISSR markers after applying other cryogenic techniques, as well as in the untreated control (Kulus 2020c). Similar variability induced by cryo-treatment was detected in *Hladnikia pastinacifolia* Rchb. (Ciringer et al. 2018) and several other plant species (Kulus and Mikuła 2016). Therefore, it can be concluded that this phenomenon is not extremely rare. Interestingly, polymorphic bands detected in the genotype obtained after cryopreservation were also identified in one

control plant (Kulus 2020c). This may indicate the presence of so-called hot spots in the genome of bleeding heart, which are particularly prone to mutations (Rogozin and Pavlov 2003). The detected polymorphisms in the non-cryopreserved control object indicate that long-term storage of *L. spectabilis* genetic resources should be performed under cryogenic conditions.

Application of plant extracts and gold nanoparticles in cryopreservation of *L. spectabilis* shoot tips

The effectiveness of cryogenic techniques can be modified using unconventional supplements, although knowledge in this area is limited. Kulus and Miler (2021) verified the usefulness of plant extracts in the cryopreservation of bleeding heart 'Gold Heart' and 'White Gold'. For this purpose, coconut, oat, rice, or sesame extract was added to the preculture medium during the first stage of the encapsulation-vitrification cryopreservation procedure. The use of extracts did not improve the survival of shoot tips stored in liquid nitrogen, and oat and sesame extracts even lowered the value of this parameter (Kulus and Miler 2021). This may result from the diverse fatty acid profile in individual extracts. The saturation level of fatty acids affects their physicochemical properties, and thus also the uptake of nutrients and cryoprotectants from the medium by explants (Meï et al. 2015). Despite the lack of improvement in tissue survival, coconut extract stimulated more intensive proliferation and shoot development after thawing the explants, indicating its usefulness in further research. This can be explained by the fact that coconut extract is rich in endogenous cytokinins and simple sugars (participating, among others, in the formation of so-called biological glass in the vitrification process) (Yong et al. 2009). Despite their potentially valuable effect, optimizing the concentrations and doses of natural plant extracts in plant tissue culture presents significant challenges due to the variability in phytochemical composition and biological activity among different plant sources. Additionally, the effects of these extracts can be influenced by factors such as the specific plant species, developmental stage, and culture conditions, making it difficult to establish standardized protocols for effective application (Hamdeni et al. 2022).

The effectiveness of cryopreservation can be improved using nanoparticles. One of the unique properties of nanoparticles is their high thermal conductivity (Li et al. 2005), which is crucial for the success of cryopreservation at the stage of freezing and thawing tissues. In the study by Kulus and Tymoszuk (2021) and Kulus et al. (2024), shoot tips of bleeding heart 'Gold Heart' and 'Valentine' were subjected to cryopreservation using the encapsulation-

vitrification method. Gold (AuNPs), silver (AgNPs), or zinc oxide (ZnONPs) nanoparticles were added at various concentrations to the preculture medium, or to the protective alginate coating, or to the *post*-freezing growth medium. To exclude the possibility of undesirable interactions that could disturb the unambiguity of the effect of the studied factor, the research included a cryotreated positive control (treated only with nanoparticle synthesis stabilizer), a cryotreated negative control (not treated with either stabilizer or AuNPs), and a standard not subjected to cryopreservation. Plants recovered from explants stored in liquid nitrogen were subjected to extensive stability analysis.

The addition of AuNPs or ZnONPs (13 nm in diameter) or AgNPs (at 6 nm) at low concentrations to the alginate bead significantly increased the effectiveness of cryopreservation (over 20% increase in explant survival compared to the untreated control), without affecting the genetic stability of plants (Kulus and Tymoszuk 2021; Kulus et al. 2024). This was probably due to the accelerated rate of cooling and thawing of tissues provided by nanometals (Vanzha et al. 2016). Supplementation of preculture medium with NPs did not cause an improvement in explants viability, although the produced shoots were significantly longer (Kulus et al. 2024). Conversely, the addition of nanoparticles to the *post*-freezing recovery medium had a negative impact on the survival of bleeding heart shoot tips. The identification of structural-metabolic changes in the *in vitro* cultures suggests that this effect resulted from the disruption of cell membrane integrity and the induction of oxidative stress in cells by aggregating nanoparticles. The negative impact of AuNPs intensified with the increase in their concentration in the medium. Additionally, it was found that the change in biochemical activity of cells and the morphogenetic response of explants (especially the rooting efficiency of plants and root parameters) depended on the moment of nanoparticle application (before or after liquid nitrogen treatment) (Kulus and Tymoszuk 2021). Generally, however, the research hypothesis on the usefulness of nanoparticles in cryopreservation was confirmed, which has great significance and can be used by a wide range of scientists. A comparison of the efficiency of various cryopreservation protocols is given in Figure 3. The obtained results also emphasize the validity of using nanoparticles in the production of artificial seeds. Interestingly, it was found that under suboptimal conditions, nanoparticles exhibited genotoxic properties (Kulus and Tymoszuk 2021). This suggests that at sufficiently high concentrations, NPs can be used as a chemical mutagen useful in bleeding heart breeding programs.

Enzymatic and non-enzymatic cellular responses to *in vitro* culture conditions

The metabolic activity of biological material is among the most critical factors determining the efficiency of *in vitro* systems, especially with medicinal crops. *In vitro* cultivation of *L. spectabilis* could have significant potential for the overproduction of valuable medicinal metabolites, particularly through the application of various elicitors that enhance biosynthetic pathways (Fazili et al. 2022). By optimizing culture conditions and utilizing substances like auxins, cytokinins, or nanoparticles, researchers could successfully increase the concentration of key compounds, such as tannins or antioxidants (Thakur et al. 2019). Moreover, the metabolic activity of plants, and their capability to produce primary and secondary metabolites, affects micropropagation and cryopreservation efficiencies.

Chlorophyll content, as the main component of chloroplasts, influences the efficiency of photosynthesis and the physiological activity of plants, affecting their growth *ex vitro*. However, stress conditions can lead to a reduction in this pigment, directly associated with decreased photosynthesis rates, ultimately inhibiting plant growth and development (Sherin et al. 2022). Flavonoids and polyphenols are antioxidants produced by plants mainly to protect them against stress and participate in cell detoxification by acting as metal chelators and neutralizing reactive oxygen species (ROS) (Thiruvengadam et al. 2015). Therefore, it is essential to monitor the physiological state and quality of plants both during *in vitro* cultivation and after transfer to greenhouse conditions (Halder et al. 2019).

To better understand the physiological responses of *L. spectabilis*, shoots and callus obtained *in vitro* were subjected to biochemical analysis for the content of pigments crucial for photosynthesis and non-enzymatic antioxidants (Kulus 2020a; Kulus et al. 2020; Kulus and Tymoszuk 2020). Additionally, Kulus and Tymoszuk (2021) and Kulus et al. (2022) determined the activity of selected antioxidant enzymes in *L. spectabilis* plants. This provided valuable information on the tolerance levels of *L. spectabilis* to specific physical and chemical factors and the mechanisms activated in response to severe abiotic stress.

Effect of plant growth regulators and nanoparticles on the content of metabolites in shoots

The study demonstrated a significant impact of *in vitro* culture conditions on the metabolite content in *L. spectabilis* shoots, with this effect depending on the cultivar and the treatment method. Auxin IAA stimulated the biosynthesis of chlorophyll and carotenoids in the 'Gold Heart' cultivar, while none of the classic growth regulators increased the content of these pigments in 'White Gold' plants (Kulus 2020a), highlighting the difficulty of growing this cultivar *in vitro*. In some experimental objects (especially in the presence of BA and KIN), a decrease in photosynthetic pigment content in shoots was observed, particularly if callus formed at their base (Kulus et al. 2020). This is surprising, as cytokinins typically participate

in plastid development and differentiation, and pigment biosynthesis (Dobránszki and Mendler-Drienyovszki 2014). The treatment method significantly influenced the anthocyanin and total polyphenol content in shoots, while it had a lesser effect on other flavonoid compounds.

On the other hand, it was found that dehydration (PVS treatment) and cryopreservation (regardless of the technique) resulted in a decreased synthesis of chlorophylls, confirming the high lability of this pigment group. Production of anthocyanins was more varied and dependent on the protocol used (Kulus 2020c).

Nanoparticles have been shown to significantly influence the production of metabolites in *L. spectabilis*. Gold nanoparticles were found to act as elicitors of tannin biosynthesis in *L. spectabilis* shoot cultures (Kulus et al. 2022). Moreover, Kulus et al. (2024) reported that the incorporation of AuNPs, AgNPs, and ZnONPs into the preculture medium during the encapsulation-vitrification cryopreservation protocol enhanced the biosynthesis of primary and secondary metabolites, including pigments such as chlorophyll and carotenoids, in a cultivar-specific manner, with notable increases observed in the 'Gold Heart' cultivar. Nanoparticles can, therefore, serve as effective elicitors to boost metabolite production.

Effect of plant growth regulators on the content of metabolites in callus

The biochemical composition of callus depended on the type of explant from which it formed (Kulus and Tymoszuk 2020), consistent with reports from other authors (Hussain et al. 2012). In *L. spectabilis*, callus regenerating from petioles was the most abundant in carotenoids and anthocyanins, while callus from whole leaves contained the least pigments, suggesting a positive impact of stress (in this case, mechanical damage to the explant) on the biosynthesis of these compounds. The auxin-to-cytokinin ratio in the medium partially influenced the chemical profile of callus (i.e., chlorophyll and carotenoid content) but did not determine anthocyanin and total polyphenol content. Generally, callus formed on a medium with NAA contained the highest concentrations of the studied metabolites. This auxin can thus be used as an elicitor to control the overproduction of desired organic compounds in *L. spectabilis* culture. Interestingly, a negative correlation was observed between the biochemical compound content in the callus and the efficiency of somatic embryogenesis. Conversely, a high frequency of non-embryogenic callus formation was associated with higher concentrations of photosynthetic pigments, anthocyanins, and polyphenols (Kulus and Tymoszuk 2020). This suggests that the chemical composition can be used as a marker of the embryogenic potential of cells and *vice versa*. A positive correlation was also found between the content of all studied chemical groups in *L. spectabilis* callus, allowing for the

simultaneous overproduction of these substances. Comparative analyses, however, showed significant differences in pigment content between callus and shoots (Kulus 2020a).

Enzymatic activity of plants in tissue culture systems

The unnatural conditions in the *in vitro* culture can lead to the release of ROS, causing lipid peroxidation and disturbances in the redox state of cells, resulting in oxidative stress (Timoteo et al. 2019). Enzymatic antioxidant mechanisms catalyze the breakdown of ROS, and changes in enzyme activity are considered biological markers of oxidative stress (Homaee and Ehsanpour 2016). Colorimetric and kinetic reaction studies allowed for understanding the enzymatic response of *L. spectabilis* cells subjected to various stressors (Kulus and Tymoszuk 2021; Kulus et al. 2022). Both in cryopreservation (Kulus and Tymoszuk 2021) and induced mutagenesis studies (Kulus et al. 2022), the highest increase in activity was observed for guaiacol peroxidase (GPOX), while the lowest was for glutathione reductase (GR). Therefore, GPOX is considered the most sensitive marker of oxidative stress in *L. spectabilis*, being the most involved in the plant's defense mechanism. Despite long-term exposure to nanoparticles, this factor generally caused the least changes in enzyme activity compared to other, more short-term stressors (Kulus et al. 2022). This underscores the suitability of using AuNPs in *L. spectabilis in vitro* systems.

Somaclonal variation and induced mutagenesis in expanding the genetic variation in bleeding heart

Breeding programs conducted in the 20th and 21st centuries have led to the creation of new cultivars of bleeding heart, although their number is still limited (Hodges 2012). In Korea, effort is made to develop commercial cultivars of this species through transgenesis (Lee and Lee 2003). However, genetic engineering is a complex, time-consuming, and costly method of plant improvement, especially in the case of bleeding heart, for which the genome size was not even known until 2022. This method is also subject to public debate. The use of somaclonal variation induced by *in vitro* culture conditions can be a cheaper, though equally effective approach (Miler and Zalewska 2014; Duta-Cornescu et al. 2023).

In the study performed by Kulus et al. (2021), it was found that bleeding heart is susceptible to somaclonal variation induced by the simultaneous presence of auxins and cytokinins in the culture medium. Variation at the DNA sequence level was detected in 36.8% and 69.1% of plants by SCoT and RAPD markers, respectively. It was probably associated with the development of genetically unstable callus at the base of shoots or exposure of plants to intensively secreted polyphenols into the medium. Additional cluster analyses and

calculated genetic distance between individuals considered most of this variation insignificant; however, four newly obtained genotypes differed significantly from the control. Due to the lack of evident phenotypic changes, the use of more "aggressive" agents for breeding purposes was indicated (Kulus et al. 2021).

Classical mutation breeding, based on random induction of mutations, is an attractive alternative to costly and difficult genetic engineering procedures (Miler et al. 2021). Mutagenic factors used in plant breeding can be classified as chemical and physical. Among chemical mutagens, ethyl methanesulfonate (EMS) is most commonly used (Shelake et al. 2019); however, recent studies have shown that nanoparticles can also be effectively used to induce genetic variability in plants due to their ease of absorption by cells and interaction with proteins and DNA (Mehrian and De Lima 2016; Tymoszuk and Kulus 2020). Physical mutagens, such as X-rays or gamma radiation, are more environmentally friendly than chemical mutagens, as their use does not generate harmful waste. Unfortunately, the availability of irradiation devices is a limiting factor for plant breeders, as these devices are usually owned by national atomic energy agencies or medical/scientific institutes (Miler et al. 2021). A cheap source of electromagnetic radiation useful in inducing variability can also be a classic microwave oven. Microwaves have been used in mutation breeding of *Chrysanthemum* × *morifolium* (Ramat.) Hemsl. (Miler and Kulus 2018).

In the study by Kulus et al. (2022), the effect of gold nanoparticles (AuNPs), microwaves (non-ionizing radiation), and X-rays (ionizing radiation), applied in various doses, was investigated on plant acclimatization efficiency and the possibility of using these factors in mutation breeding and inducing variability at the genetic and phenotypic level in bleeding heart.

It is worth emphasizing the high survival rate of primary explants in the *in vitro* culture, which ranged from 86% to 100% (Kulus et al. 2022). This is significantly higher than in other species treated with mutagens (Tallón et al. 2015) and may indicate high resistance of *L. spectabilis* to this type of stress. Bleeding heart may therefore constitute a valuable source of genes in breeding programs based on crossing, hybridization, and transgenesis. It was found that plants from the untreated control and plants obtained after the longest period of microwave irradiation more often died during acclimatization than those from other experimental treatments. Control plants were also of noticeably poorer quality during further greenhouse cultivation (Kulus et al. 2022). The hormetic response may therefore be a strategy by which *L. spectabilis* acclimates to more difficult conditions, leading to speciation in the evolutionary process (Małkowski et al. 2020).

Cytometric analyses allowed to determine that *L. spectabilis* has a very small genome (1281 Mbp; 1.314 pg/2C DNA), according to the classification by Soltis et al. (2003), which makes it a good model species for experimental biology research, concerning, for example, directions of evolution. No changes in ploidy were detected among the studied plants, but in several individuals treated with X-rays, nanoparticles, or microwaves, significant changes in nuclear DNA content were found (Kulus et al. 2022). In most cases, there was a decrease in DNA content (most likely due to deletions), and in one case, an increase in the value of this parameter (which can be explained by the transition of somatic cells into endocycle). A similar phenomenon was observed, for example, in chrysanthemum (Miler et al. 2020). The greatest fluctuations in DNA content were found after applying the highest dose of X-rays, suggesting that this factor affects bleeding heart plants to the greatest extent.

The effectiveness of the applied mutagens in inducing variability in *L. spectabilis* was confirmed by SPAR (single primer amplification reaction) genetic analyses (Kulus et al. 2022). This confirms the usefulness of this group of markers in poorly studied species. PCR-based genotyping methods are still widely used to assess plant genetic variability due to their simplicity and versatility (Nadeem et al. 2017). Mutations were detected in 7.5% of plants by DAMD (Directed Amplification of Minisatellite DNA (Heath et al. 1993)), RAPD, and SCoT markers, but not by ISSRs. Therefore, the discriminatory power of the studied molecular marker systems in *L. spectabilis* can be arranged in the following order: ISRR<SCoT<RAPD<DAMD (Kulus et al. 2022). The lack of species-specific primers confirmed the usefulness of primers previously used in other botanical families. Interestingly, most polymorphisms were detected by the DAMD primer 5'-AATGTGGGCAAGCTGGTGGT-3' (Seyedimoradi et al. 2012), although the PCR thermal profile for this marker system was the most difficult to optimize. Phenotypic changes concerning leaf shape were identified in plants irradiated with X-rays or, less frequently, in plants treated with AuNPs. Despite the observed changes in DNA sequence, no permanent phenotypic changes were found in objects treated with microwaves, suggesting that this is the least effective of the studied mutagens (Kulus et al. 2022). These experiments create perspectives leading to obtaining new cultivars of *L. spectabilis* plants with increased horticultural and/or phyto-pharmaceutical potential.

Conclusions

Research indicates that in bleeding heart, the activation of axillary buds *in vitro* (especially in the presence of KIN in the medium) and the induction of indirect somatic embryogenesis (in

the presence of PIC) are significantly easier to achieve than the regeneration of adventitious shoots. There are evident cultivar differences within the species. The 'Valentine' cultivar is the easiest to culture *in vitro*, while 'White Gold' is the most challenging. Since classical growth regulators can cause anatomical, physiological, and biochemical disturbances in bleeding heart, it is advisable to search for new substances stimulating morphogenesis *in vitro*. Examples of such substances may include nanoparticles or substances of natural origin. The negative effects of synthetic growth regulators can be avoided by supplementing the medium with coconut or rice extract (low in polyphenols and high in saturated fatty acids), depending on the preferences of individual cultivars. The addition of gold nanoparticles, on the other hand, positively affects shoot proliferation and the quality of the obtained plantlets, which are characterized by higher survival rates after transferring to *ex vitro* conditions. Simultaneous overproduction of bleeding heart metabolites can be achieved using exogenous auxins (IAA, NAA) and gold nanoparticles. Analysis of cellular enzymatic activity identified guaiacol peroxidase as the most sensitive marker of oxidative stress in *L. spectabilis* caused by water deficit or thermal factors. The most stable among the studied catalytic proteins under stress conditions is glutathione reductase. It was demonstrated that cryogenic techniques based on explant encapsulation are the most effective in terms of biological material viability, its further growth, and genetic stability. Nanomaterials in low concentrations can increase the effectiveness of cryogenic techniques by over 20%. This discovery may be groundbreaking for the further development of cryobiology, although the final effect depends also on their size. The addition of coconut extract to the medium also positively affects shoot proliferation after "thawing" of explants. On the other hand, sesame extract can be used as a natural retardant, for example, in slow-growth plant culture. The effectiveness of tested mutagens in mutation breeding of bleeding heart can be ranked as follows: microwaves < AuNPs < X-rays. Although individual SPAR marker systems have certain limitations, the collective data obtained from several types of systems provide a comprehensive description of genetic variability within the species.

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Fig. 1. Four commercial cultivars of *L. spectabilis*: ‘Alba’ (A), ‘Gold Heart’ (B), ‘White Gold’ (C), and the most recent one ‘Valentine’ (D) cultivated at the Bydgoszcz University of Science and Technology, Poland.

Fig. 2. *In vitro* growth of single-node explants of *L. spectabilis* on the MS medium devoid of plant growth regulators (A), the presence of auxins and cytokinins (PGRs) stimulates the secretion of harmful phenolics into the medium (B), gold nanoparticles (AuNPs) in the culture medium stimulate the most abundant growth of good-quality shoots (C), non-meristematic explants (leaves and internodes) regenerate only embryogenic callus (D), complete micropropagated plants grown *ex vitro*.

Fig. 3. Comparison of the effectiveness of various cryopreservation protocols in *L. spectabilis*: V – vitrification, D-V – droplet-vitrification, E-V – encapsulation-vitrification, E-D – encapsulation-dehydration.

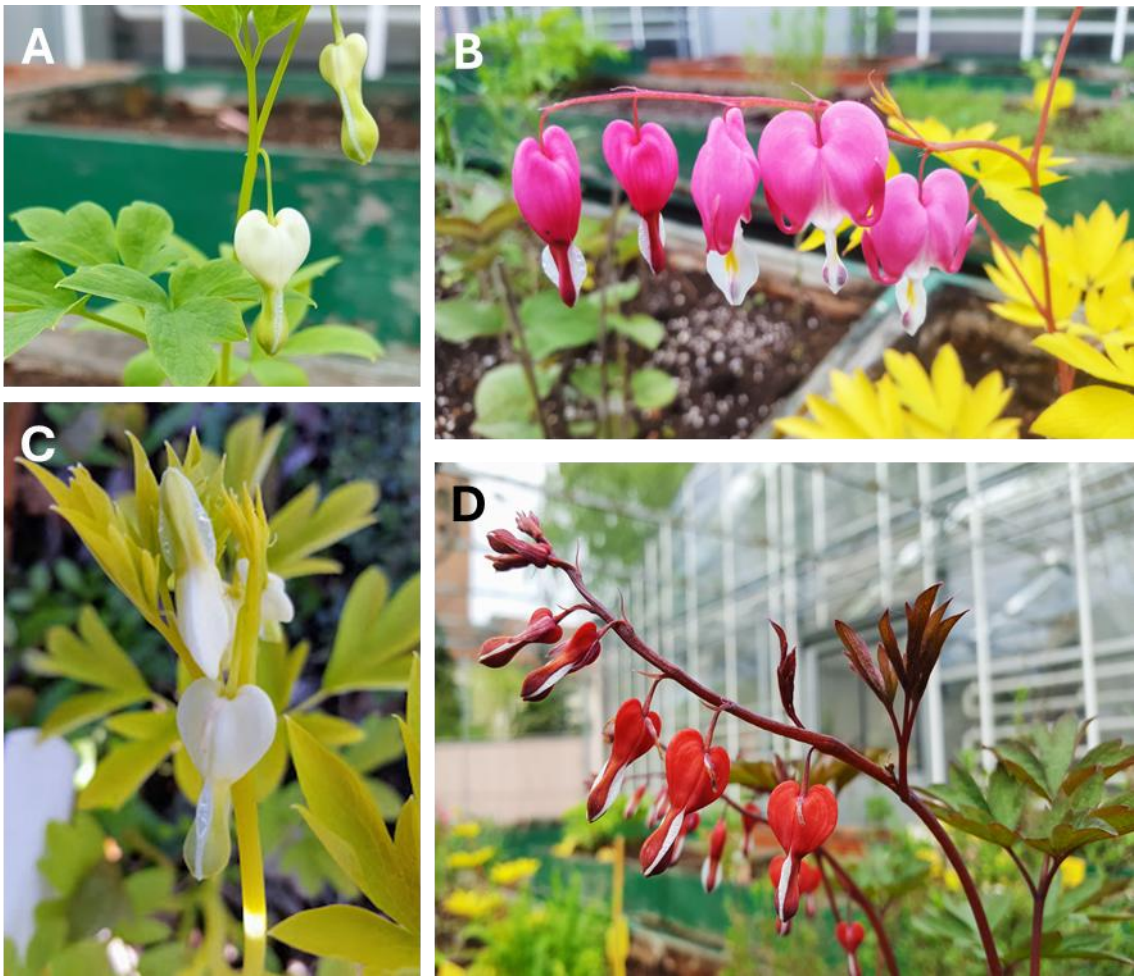
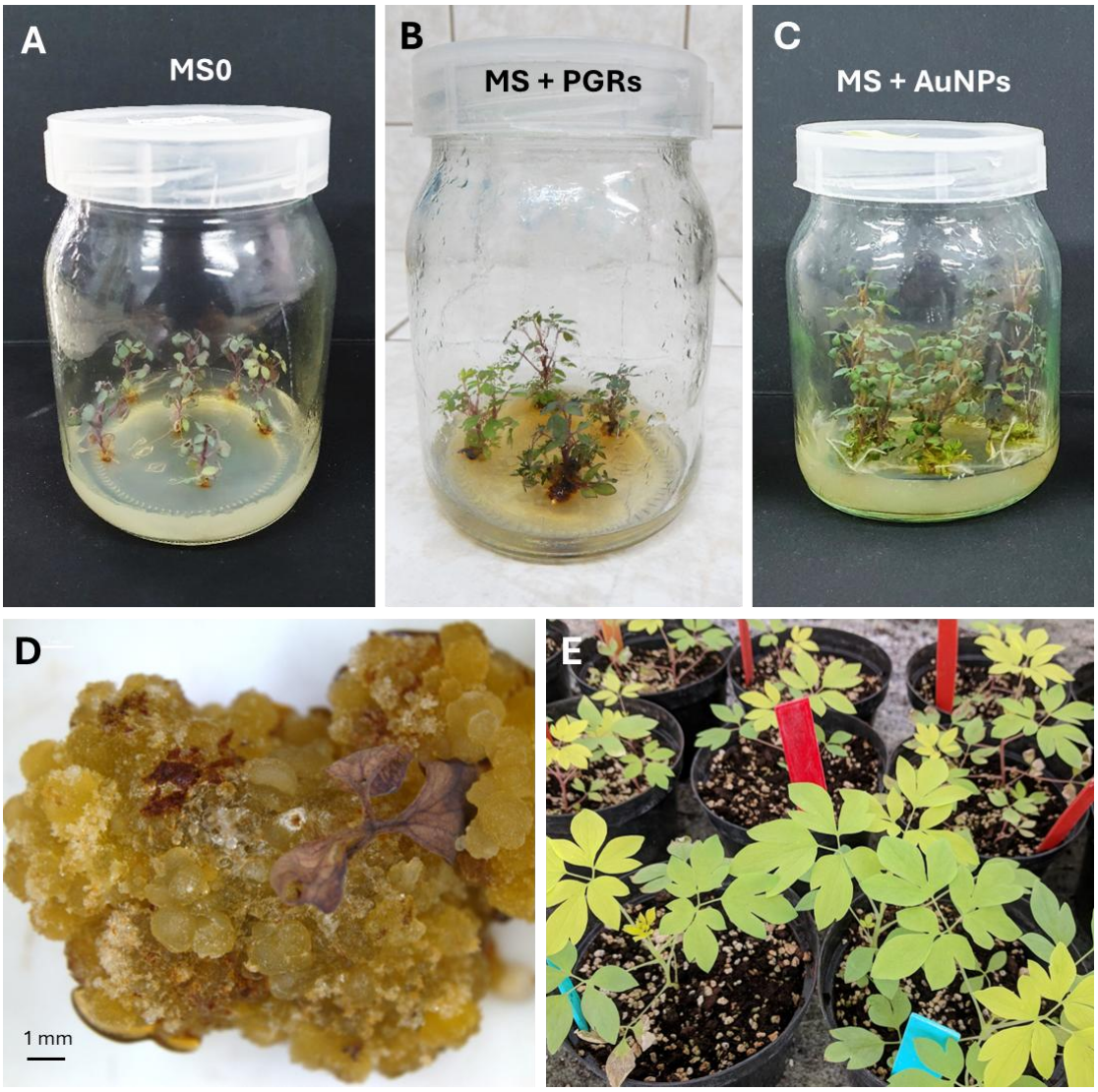


Fig. 1



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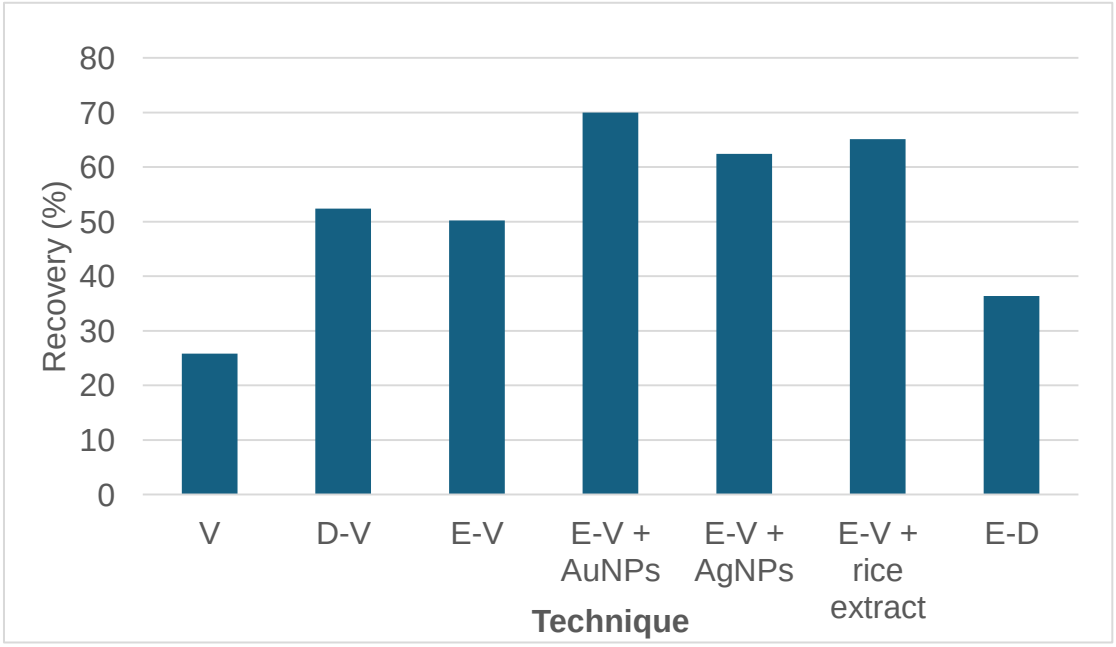

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