

Research Paper

In vitro micropropagation of *Dianthus plumarius* subsp. *praecox*, a wild carnation species, for ornamental horticultural and gene conservation purposes

Pál Szarvas^{a,1}, Dóra Farkas^{a,1}, Judit Csabai^b, Angéla Kolesnyk^c, Judit Dobránszki^{a,*}

^a Centre for Agricultural Genomics and Biotechnology, Faculty of the Agricultural and Food Science and Environmental Management, University of Debrecen, P.O. Box 12, H-4400 Nyíregyháza, Hungary

^b Institute of Engineering and Agricultural Sciences, University of Nyíregyháza, Sóstói str. 31/b, H-4400, Nyíregyháza, Hungary

^c Uzhhorod National University, Department of Genetics, Plant Biology and Microbiology, 3 Narodna Square, Uzhhorod 88000, Ukraine

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ABSTRACT

Carnation is important floral crop. The introduction of wild carnation species is carried out in parallel with research on existing species and varieties. *Dianthus plumarius* subsp. *praecox* is a wild species with an extremely high germplasm conservation importance. The most important step in the study of its cultivation is to propagate it *in vitro* under conditions of minimum impact on nature. Therefore, present work aimed to develop an *in vitro* micropropagation protocol for this species. Different types of cytokinins, such as 6-benzylaminopurine, 6-benzylaminopurine riboside, meta-topolin, N-(2-chloro-4-pyridyl)-N'-phenylurea, kinetin, N⁶-(2-isopentenyl) adenine, zeatin and adenine sulfate, was applied for the *in vitro* multiplication. Cytokinins were applied alone in a concentration of 4.5 μ M, or in the combination of 2.25 μ M BA and 2.25 μ M KIN. Their effect on the *in vitro* shoot multiplication and their after-effects on the *in vitro* rooting were tested. Based on the present detailed study, a micropropagation protocol was developed, where high shoot multiplication rate (4.7 ± 0.4 shoot/explant with 25.1 ± 1.4 -mm-long shoots) could be achieved using zeatin as cytokinin source in the shoot multiplication medium. For successful *in vitro* rooting 6-benzylaminopurine-containing medium was applied for 3 weeks after shoots multiplication phase on zeatin-containing medium, then shoots were conditioned for 2 weeks on plant growth regulator free medium directly before rooting. With this treatment hyperhydration of *in vitro* shoots could be decreased from 91 % to 12 % and high frequency (86.7 %) *in vitro* rooting was gained. Rooted *in vitro* shoots were successfully acclimatized in 71 %.

1. Introduction

The *Dianthus* genus from the family *Caryophyllaceae* consist of 400 to 600 species of perennial flowers with spectacular color combinations ranging from yellow, red to deep purple. It is known, that some species of *Dianthus* produce important secondary metabolites which are useful in agriculture or in human/animal therapy (Matern, 1994). *Dianthus superbus* L. and *D. chinensis* L. are used in insecticidal pesticides and also used as medicine to reduce fevers and diuresis (Yoon et al., 2019; Zhang

et al., 2019). Some *Dianthus* species are able to reduce or eliminate pollutants, for instance chlorine and sulfur dioxide can be absorbed by *D. barbatus* L. making the species a great air purifier (Wang et al., 2021). *D. plumarius* L., *D. barbatus* L., *D. giganteiformis* subsp. *kladovanus* (Degen) Soó. has a significant application value as an environmental protection against soil erosion and greening plant due to their tolerance to abiotic stress and great reproduction rate (Wang et al., 2021; Jean-Vasile et al., 2013).

The carnation family is one of the most important groups in

Abbreviations: 2-iP, N⁶-(2-isopentenyl) adenine; 4CPPU, N-(2-chloro-4-pyridyl)-N'-phenylurea; AD, adenine sulfate; BA, 6-benzylaminopurine; BAR, 6-benzylaminopurine riboside; KIN, kinetin; MTP, metatopolin [6-(3-hydroxybenzylamino) purine]; NAA, 1-Naphthaleneacetic acid; ZEA, Zeatin [N⁶-(4-hydroxy-3-methylbut-2-enyl) adenine].

* Corresponding author.

E-mail addresses: szarvas.pal@agr.unideb.hu (P. Szarvas), farkas.dora@agr.unideb.hu (D. Farkas), csabai.judit@nye.hu (J. Csabai), alexkolesnyk@online.ua (A. Kolesnyk), dobranszki@freemail.hu (J. Dobránszki).

¹ These authors share first authorship.

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ornamental horticulture. The International Dianthus Register includes approximately 30,000 *Dianthus* cultivars (Leslie 2016). Micropropagation is a technique commonly employed in the cultivation of *Dianthus* species to prevent the spread of several diseases into the stand while also producing a large and homogenous stock. In addition to the endogenous, antifungal-based resistance modulation (Shah et al., 2020), the *in vitro* propagated cuttings can be safely protected from the most common phytopathogens like viruses such as Carnation Latent Virus (CLV), Carnation Etched Ring Virus (CERV), Chrysanthemum Virus B (CVB), Chrysanthemum Aspermy Virus (CAV) (Gautam et al., 2019) and vascular wilt caused by *Fusarium oxysporum* f. sp. *dianthi* (Romero-Rincón et al., 2021). In addition to plant protection problems stem strength, vase life, and fragrance are critical issues. The introduction of wild carnation species into breeding can help to replace lost or missing genetic features in present varieties, such as scentloss during breeding (Gatt et al., 1998).

The white-flowered carnations of the Carpathians and the Carpathian Basin are divided into numerous small species that alternate in different mountain ranges (Farkas 1999; Király 2009). Barina et al. (2020) distinguish 4–5 taxa of *D. plumarius* in the region. *D. plumarius* subsp. *praecox* (Willd. ex Spreng.) Domin (Kliment et al., 2016) is a wild perennial plant species and is on the Carpathian List Of Endangered Species (Witkowski et al., 2003). Its narrow distribution, in Hungary (Békkő in the Bükk Mountains) and Slovakia (Pénzes-Kónya et al., 2014), is also indicated by the name *Dianthus hungaricus* Pers., which is used as a synonym in some literature (Fassou et al., 2022). *D. plumarius* and its varieties are cultivated in horticulture, but this particular species of limited occurrence has not yet been published, although it can be a wonderful scented plant for rockery compositions and landscape gardens without any breeding intervention.

There are plenty of studies relating to the optimal parameters of *in vitro* micropropagation of the *Dianthus* genus. Regarding the initiation stage for seeds hormone-free MS (Păunescu and Holobiuc, 2003; Catană et al., 2013), or MS subsidized with Gamborg vitamins were also a great option for some taxa (Cristea et al., 2006). The most widely used media for the multiplication stage were MS supplemented with 4.4 μM 6-benzylaminopurine (BA) and 0.54 μM 1-naphthaleneacetic acid (NAA) alone (Jarda et al., 2011; Păunescu and Holobiuc, 2003; Catană et al., 2013) or combined with 4.7–3.1 μM kinetin (KIN) and other plant growth regulators (PGRs) (for example 2,4-D and GA₃). In some cases, the usage of Gamborg vitamins instead of MS vitamins was preferred (Holobiuc and Blindu, 2006, 2007). For the rooting stage, the culture medium containing MS supplemented with 2.3 μM KIN or 4.4 μM BA and 0.54 μM NAA performed exceptionally well with an adequate root formation and a 60–80 % acclimatization rate (Cristea et al., 2010, 2002; Jarda et al., 2014).

According to Pence (2011), there are at least 5000 endangered species worldwide that will need *in vitro* methods for gene conservation, reintroduction in nature, research and education purposes, or commercial use. *In vitro* methods include both short-, medium-, (working, active collections) and long-term (base, seed collections) plant cultures, that may be efficient in case of limited initial plant materials, supplementing conventional *in situ* conservation strategies (Breese, 1989; Malhotra et al., 2019). Short-term *in vitro* plant cultures have four stages. The success of the first, initiation stage depends on choosing the right type of plant material (shoot tip, nodal segments, seeds etc.), at the optimal physiological developmental phase and in adequate quantity. Furthermore, choosing the appropriate disinfectant, disinfection method and a suitable medium are essential for a successful establishment of *in vitro* culture (Păunescu, 2009). The PGRs are crucial for the following multiplication stage, where the main goal is the amplification of the number of shoots. The third, rooting stage is fundamental for increasing the plantlets' survival rate during acclimatization (Radomir et al., 2023). The fourth phase is acclimatization, where the type of substrate and training method are necessary for an efficient survival rate greater than 50 % (Radomir et al., 2023.) *In vitro* plant

micropropagation gives a possibility to keep alive special lines with genetic identity for conservation purposes, to multiply endangered plant species for repatriation, or producing large plant biomass for yielding secondary metabolites (Crouch and van Staden, 1993; Khatun et al., 2013).

Although there appear to be many research papers regarding the micropropagation of endangered species of the *Dianthus* genus, however, the *D. plumarius* taxon is an exception (Radomir et al., 2023). Loss of natural habitats of this subspecies led to significant population declines, furthermore, carnation seeds are often damaged by herbivorous insects. As a consequence, their seed yield reduced below an ecological level where the maintaining of this subspecies in the wild is hardly or not even possible. Therefore, the aim of this study was to establish an optimal *in vitro* micropropagation protocol for *D. plumarius* subsp. *praecox* (Willd. ex Spreng.) Domin.

2. Materials and methods

2.1. Culture establishment

Seeds of *Dianthus plumarius* subsp. *praecox*. (Willd. ex Spreng.) Domin. were originated from the Botanical Garden of the University of Eszterházy Károly in Eger (original habitat: Békkő of Bükk Mountains). The Botanic Garden stock did not receive any pretreatment or nutrient supplementation. The plants were maintained in the garden in an environment imitating the natural location. The seeds were harvested in the summer of 2022. *In vitro* culture establishment occurred in March of 2023 in the plant tissue culture laboratory of the Centre for Agricultural Genomics and Biotechnology of University of Debrecen. The disinfection of seeds was carried out according to Cseh et al. (2023). After that, the sterilized seeds were put onto solidified, whole strength MS medium (Murashige and Skoog, 1962) supplemented with 3 % sucrose and 6.5 g l⁻¹ agar (Merck - Sigma-Aldrich, A1296, microbiology tested, suitable for plant cell culture, suitable for cell culture, powder) without any plant growth regulators (PGRs) for germination. Germination occurred at 23 $\pm$ 2 °C and at a light intensity of 80–106 $\mu\text{mol s}^{-1} \text{m}^{-2}$. Five seeds per jar (390 ml) were cultured together. 90 % of the seeds germinated within 2 days. One-nodal segments were excised from 3-week-old seedlings, and transferred onto MS medium, supplemented with 3 % sucrose and 6.5 g l⁻¹ plant agar. The pH was set to 5.8 prior to the sterilization process carried out by an autoclave at 121 °C, 1.2 bar pressure. Cultures were covered with drilled out steel caps equipped with sponge plugs to ensure safe ventilation. In each jar, 70 ml medium was poured into. All cultures (including seedlings and plants) were kept in a culture room at a temperature of 23 $\pm$ 2 °C and a 16/8 photoperiod provided by 1:1 mix of daylight and warm white fluorescent lamps in a vertical position which resulted in a light intensity of 80–106 $\mu\text{mol s}^{-1} \text{m}^{-2}$. Culture period lasted 3 weeks. This process was carried out several times in order to obtain a suitable amount of plant material.

2.2. Shoot multiplication and elongation

Nodal segments of 10 mm were put onto MS medium supplemented with 3 % sucrose, 6.5 g l⁻¹ plant agar, 0.54 μM 1-naphthaleneacetic acid (NAA) and 8 types of cytokinins (CKs), such as: 6-benzylaminopurine (BA), 6-benzylaminopurine riboside (BAR), kinetin (KIN), metatoplin (MTP), N-(2-chloro-4-pyridyl)-N'-phenylurea (4CPPU), N⁶-(2-isopentenyl) adenine (2-iP), zeatin (ZEA), adenine sulfate (AD) applied alone in a concentration of 4.5 μM , or applied in the combination of 2.25 μM BA and 2.25 μM KIN (BA+KIN). All types of PGRs were added to the medium before autoclaving. PGR-free MS medium served as a control. The multiplication phase lasted 3 weeks. Shoot clusters from the multiplication phase were transferred to a PGR-free MS medium (elongation medium). The elongation phase lasted 2 weeks.

2.3. In vitro rooting and acclimation

Elongated shoot clusters were separated into individual shoots that were then cultured for two weeks on rooting medium containing full-strength MS medium supplemented with 2 % sucrose, 6.5 g l⁻¹ plant agar and 0.54 μM NAA.

Rooted plantlets were removed carefully from the solid medium and thoroughly washed with tap water, then transferred into pots filled with commercial planting soil (Mr. Garden, Agro CS Hungary Ltd.) moistened with tap water. Pots were covered with transparent plastic caps for two weeks to maintain adequate humidity. The acclimation process lasted 4 weeks in a climate room, at a temperature of 23 ± 2 °C, 16/8 h photoperiod, provided by daylight, flora and warm white fluorescent lamps at a ratio of 1:1:1 respectively, resulting in 130 μmol s⁻¹ m⁻² light intensity.

2.4. Measurements and statistical analysis

After the multiplication phase the length of shoots (SL_M), number of new shoots per explant (SN_M), node number per shoot (NN_M) and percentage of hyperhydrated shoots (H_M%) and rooting percentage (RP_M) were measured from which data the multiplication index was calculated as follows:

$$MI = \frac{SL_M \times SN_M}{NN_M} \quad (1)$$

After the elongation phase the shoot length (SL_E), percentage of hyperhydrated shoots (H_E%), and node number per shoot (NN_E) were measured. From the data, hyperhydricity restoration (HR) and elongation index (EI) were calculated as follows:

$$EI = \frac{SL_E \times HR}{NN_E} \quad (2)$$

where

$$HR = 1 - \frac{H_E\%}{H_M\%} \quad (3)$$

After the rooting phase the percentage of rooted shoots (RP_R), the number of roots per shoot (RN_R) and the length of roots (RL_R) were measured.

One-way ANOVA followed by Tukey's test at $p < 0.05$ using SPSS for Windows software (SPSS®, version 21.0) was applied for analyzing the morphological data from different phases of micropropagation.

Finally, the percentage of plantlets that acclimatized successfully and continued to develop were calculated.

3. Results

3.1. Shoot multiplication phase

At the multiplication phase, the mean shoot length (SL_M) ranged from 8.2 ± 1.3 to 25.1 ± 1.4 mm per explant. Among all treatments, ZEA showed significant ($p < 0.05$) effect on shoot length (25.1 ± 1.4 mm) resulting the highest shoots. After the ZEA treatment, the best results were achieved on BA+KIN (18.4 ± 1.2 mm), control (16.2 ± 1.7 mm) and 2-iP media (15.8 ± 1.2 mm), however, they resulted in significantly lower shoot length compared to the effect of ZEA. With regard to the number of *de novo* developed shoots (SN_M) the best results were achieved by applying BA+KIN (6.4 ± 0.5 shoots/explant). The MS medium containing ZEA gave the second maximum mean shoot number (4.7 ± 0.4 shoots/explant).

Apart from shoot length, the number of nodes (NN_M) determine the multiplication rate if using one-nodal segments to propagate. According to the data, ZEA yielded the highest number of nodes per explant (3.7 ± 0.2), but this outcome was not significantly different from the results of

Table 1

Mean values of measured parameters with standard errors at multiplication phase. Measurements are followed by letters that indicate significantly ($P < 0.05$) different values between treatments according to ANOVA and Tukey tests (abbreviations: SL_M = shoot length (mm); SN_M = new shoot number/explant; NN_M = node number/shoot; H_M% = hyperhydration percentage (%); RP_M = rooting percentage (%); MI = multiplication index; M in lower index = multiplication phase).

		SL _M	SN _M	H _M %	NN _M	RP _M	MI
1	Ø	16.2 ± 1.7c,d	2.7 ± 0.4a	13.3	2.7 ± 0.2b,c,d	60.0	16.2b
2	BA	11.1 ± 0.9a,b	4.4 ± 0.4b,c	91.1	1.6 ± 0.1a	8.8	30.5a
3	BAR	10.2 ± 0.8a,b	4.3 ± 0.5b,c	76.0	1.5 ± 0.1a	2.0	29.2a
4	KIN	13.9 ± 1.3b,c	3.0 ± 0.4a,b	32.0	2.4 ± 0.2b,c	6.0	17.4b
5	MTP	13.0 ± 1.5a,b	2.2 ± 0.3a	32.0	2.0 ± 1.5a,b	0.0	14.3b
6	4-CCPU	8.2 ± 1.3a	1.9 ± 0.3a	38.0	1.3 ± 0.2a	0.0	12.0c
7	2-iP	15.8 ± 1.2c,d	2.2 ± 0.2a	26.2	3.1 ± 0.2c,d,e	0.0	11.2c
8	ZEA	25.1 ± 1.4e	4.7 ± 0.4d	22.0	3.7 ± 0.2e	8.0	31.9a
9	AD	13.4 ± 1.2a,b	1.7 ± 0.2a	18.0	2.9 ± 0.2c,d,e	16.0	7.9d
10	BA+KIN	18.4 ± 1.2d	6.4 ± 0.5e	50.0	3.2 ± 0.2d,e	0.0	36.8a

BA+KIN (3.2 ± 0.2), 2-iP (3.1 ± 0.2), and AD (2.9 ± 0.2). The next advantageous feature from the multiplication point of view was the number of new shoots per explant (SN_M) based on which BA+KIN achieved significantly the highest number (6.4 ± 0.5), however, the percentage of hyperhydrated shoots (H_M%) were relatively high (50.0 %), too. This contradiction prompted us to look at the experiment from a different perspective. A multiplication index (MI) was created to determine the most suitable medium for the multiplication phase that encompass all the important features except hyperhydricity. In accordance with the MI the optimal multiplication medium was BA+KIN (36.8), that did not differ significantly from ZEA (31.9), BA (30.5) and BAR (29.2) (Table 1). However, as mentioned earlier, hyperhydration is not included in the index, so this factor must be taken into account separately. Among the four most suitable medium, ZEA attained the lowest hyperhydricity percentage with 22.0 %, therefore for the multiplication phase ZEA conforms the best results.

Rooting was observed on the control media (60.0 %) and on some other multiplication media containing AD (16.0 %), BA (8.8 %) ZEA (8.0 %), KIN (6.0 %) and BAR (2.0 %), surprisingly.

3.2. Elongation phase

Shoot length (SL_E) at the elongation phase ranged from 18.7 ± 2.6 mm to 47.5 ± 2.7 mm where the best results were accomplished on PGR-free medium (control). To be able to gather as many one-nodal segments as possible, not only shoot length, but the number of nodes is also important. The maximum number of nodes per shoot (NN_E) were recorded on PGR-free medium (control, 5.4 ± 0.2) but it was not significantly different from the AD (5.0 ± 0.3), ZEA (4.4 ± 0.2), 2-iP (4.4 ± 0.2) and BA (4.4 ± 0.3) treatments. Hyperhydricity encumber the excision, transplantation and cultivation of one-nodal segments. After the multiplication phase significantly more explants/shoot clusters were hyperhydrated than in the elongation phase, therefore hyperhydricity restoration were calculated. According to the results, the usage of PGR-free medium facilitated the recovery of shoot clusters in all cases except of AD, where the percentage of hyperhydricity remained the same. With the aim of choosing the most suitable medium for the micropropagation, an elongation index (EI) that contains all the before

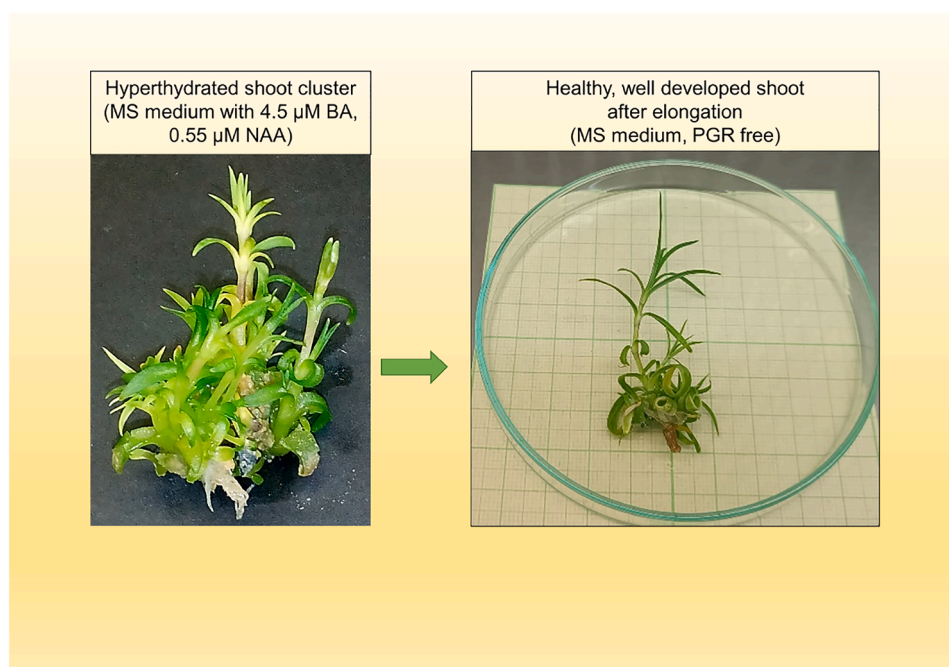


Fig. 1. Hyperhydrated shoots developed on BA-containing medium recovered in 2 weeks on PGR-free elongation medium.

Table 2

Mean values of measured parameters with standard errors at elongation phase. Measurements are followed by letters that indicate significantly ($P < 0.05$) different values between treatments according to ANOVA and Tukey tests (abbreviations: SL_E = shoot length (mm); NN_E = node number/shoot; $HM\%$ = hyperhydration percentage at multiplication phase (%); $HE\%$ = hyperhydration percentage at elongation phase (%); HR = hyperhydricity restoration; EI = elongation index; M in lower index = multiplication phase; E in lower index = elongation phase).

		SL_E	NN_E	$HM\%$	$HE\%$	HR	EI
1	Ø	$47.5 \pm 2.7e$	$5.4 \pm 0.2e$	13.3	0.0	1.0	8.8
2	BA	$30.7 \pm 2.2c,d$	$4.4 \pm 0.3b,c,d,e$	91.1	12.0	0.9	6.1
3	BAR	$25.2 \pm 2.2a,b$	$3.7 \pm 0.3b,c$	76.0	15.0	0.8	5.5
4	KIN	$23.2 \pm 1.1a,b$	$3.6 \pm 0.1b,c$	32.0	9.2	0.7	4.6
5	MTP	$23.5 \pm 2.2a,b$	$3.3 \pm 0.3a,b$	32.0	6.7	0.8	5.6
6	4-CCPU	$18.7 \pm 2.6a$	$2.6 \pm 0.2a$	38.0	6.7	0.8	5.9
7	2-iP	$28.4 \pm 1.7a,b,c$	$4.4 \pm 0.2b,c,d,e$	26.2	1.8	0.9	6.0
8	ZEA	$36.5 \pm 3.0d$	$4.4 \pm 0.2b,c,d,e$	22.0	2.5	0.9	7.4
9	AD	$36.8 \pm 2.9d$	$5.0 \pm 0.3d,e$	18.0	18.0	0.0	0.0
10	BA+KIN	$28.6 \pm 2.0a,b,c$	$4.1 \pm 0.2b,c,d$	50.0	0.0	1.0	7.0

mentioned data was created. The highest EI was recorded on the PGR-free (control, 8.8) medium, followed by ZEA (7.4), BA+KIN (7.0) and BA (6.1) (Fig. 1, Table 2). Summarizing the results on elongation medium, PGR-free medium produced significantly the highest shoots (SL_E) (Table 2), whereas all the other treatments were performing relatively poorly compared to the control group.

3.3. Rooting phase, acclimatization

After the rooting phase, ZEA performed poorly regarding the root length (4.3 ± 1.5 mm), but BA exceeded all the expectations with 33.4 ± 9.6 mm root length. The same tendency with regard to the root numbers (RN_R), where BA achieved a superb 8.3 ± 2.6 roots per explant, and the highest rooting percentage (RP_R), where 86.7 % of explants had roots, was observed (Table 3). All other CKs performed significantly worse

Table 3

Mean values of measured parameters with standard errors at rooting phase. Measurements are followed by letters that indicate significantly ($P < 0.05$) different values between treatments according to ANOVA and Tukey tests (abbreviations: RL_R = root length (mm); RN_R = root number/shoot; RP_R = rooting percentage (%); R in lower index = rooting phase).

		RL_R	RN_R	RP_R (%)
1	Ø	$14.4 \pm 2.5a$	$3.4 \pm 0.6a$	58.2
2	BA	$33.4 \pm 9.6b$	$8.3 \pm 2.6b$	86.7
3	BAR	$7.4 \pm 2.9a$	$2.3 \pm 0.8a$	32.0
4	KIN	$10.5 \pm 3.2a$	$1.6 \pm 0.5a$	26.7
5	MTP	$3.5 \pm 1.4a$	$1.9 \pm 1.1a$	32.0
6	4-CCPU	$11.7 \pm 4.9a$	$2.0 \pm 0.8a$	40.0
7	2-iP	$11.5 \pm 2.8a$	$2.7 \pm 0.7a$	36.4
8	ZEA	$4.3 \pm 1.5a$	$1.1 \pm 0.3a$	25.0
9	AD	$10.0 \pm 3.2a$	$1.8 \pm 0.5a$	25.0
10	BA+KIN	$6.6 \pm 2.3a$	$1.3 \pm 0.4a$	30.0

than BA.

After the 2-week long acclimatization process, it was determined that 71 % of the plants survived and started to develop.

4. Discussion

In the present study, it was desired that our endeavour yielded an optimal *in vitro* propagation protocol for *Dianthus plumarius* subsp. *praecox* species. A successful micropropagation depends on several crucial factors including the strategic use of growth regulators and optimal culture conditions. Although cytokinins are the main governors of *in vitro* shoot multiplication (Van Staden et al., 2008), but limited information is available on the micropropagation of this species, therefore a wide range of cytokinins in the same concentration (or combined) for *Dianthus plumarius* subsp. *praecox* were examined.

Although for shoot multiplication of related carnation species 4.4 µM BA alone or combined with KIN were most often applied (Jarda et al., 2011; Păunescu and Holobiuc, 2003; Catană et al., 2013; reviewed in Radomir et al., 2023), application of BA or BA+KIN for shoot multiplication of this species caused high hyperhydricity and low or medium shoots (11.1 ± 0.9 mm and 18.4 ± 1.2 mm, respectively) (Table 1).

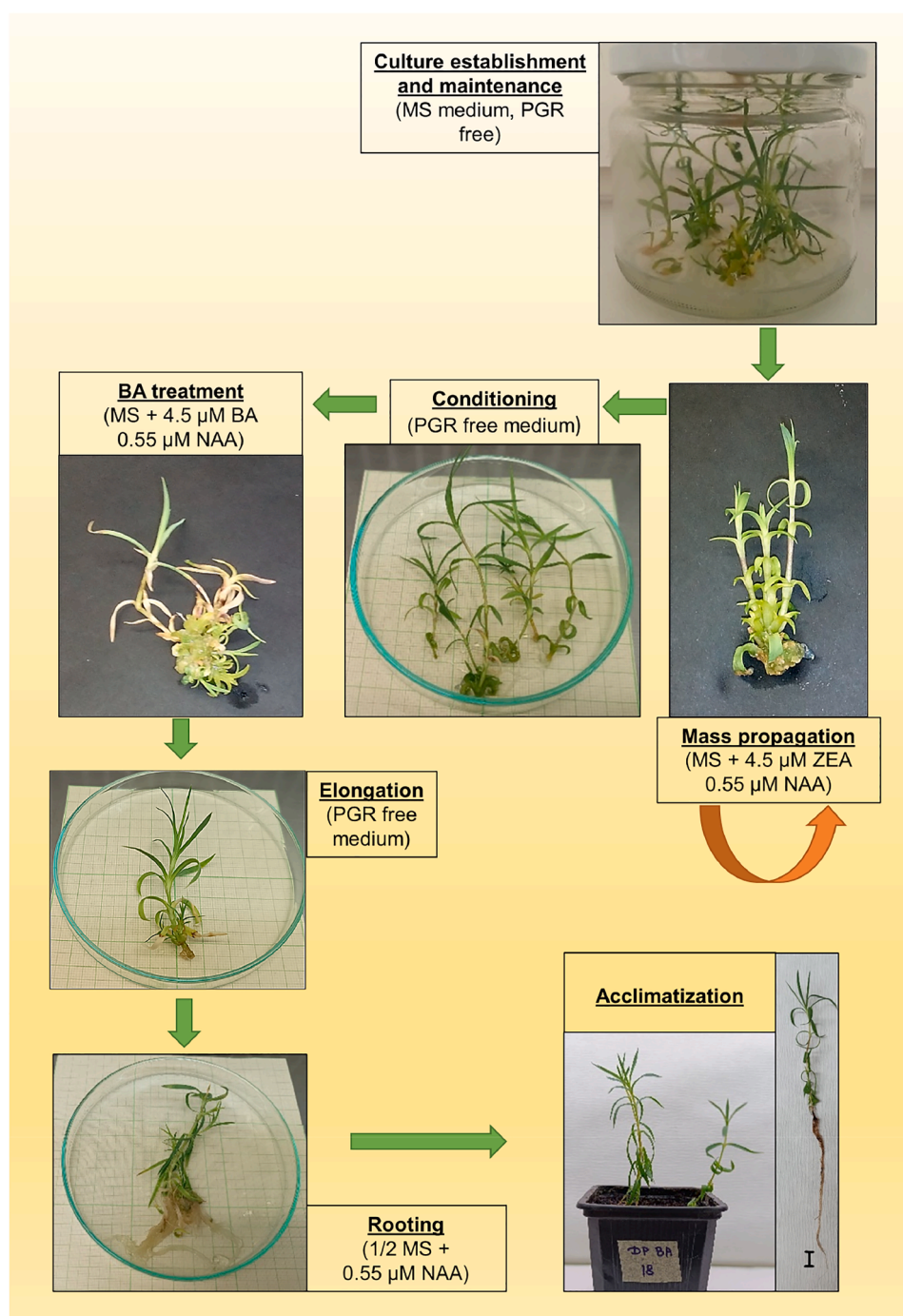


Fig. 2. *In vitro* protocol proposed for maintenance and micropropagation of *Dianthus plumarius* ssp. *praecox*.

However, the application of ZEA resulted in similar high multiplication index (31.9), as was detected for BA (30.5) and BA+KIN (36.8), and reduced hyperhydration by more than half (compared to BA+KIN) or a quarter (compared to BA). This study revealed that during the multiplication phase ZEA performed the best ($MI = 31.9$, $H_M\% = 22\%$) (Table 1), ZEA was recommended for mass propagation of *in vitro* shoots in the micropropagation protocol (Fig. 2). Our results indicate that it would be worthwhile to investigate the effect of ZEA on *in vitro* shoot multiplication of other carnation species as well.

Considering the height of the newly developed shoots, instead of excising one-nodal segments, shoot clusters were put onto the elongation medium together. In order to be able to achieve large-scale propagation of *D. plumarius*, it is advisable to take into account all the

indicators measured after elongation phase including number of nodes per shoot (NN_E), shoot length (SL_E), and hyperhydricity restoration (HR). For this purpose, an elongation index (EI) was created. The obtained results show, that the PGR-free (control group) had the highest EI (8.8), followed by ZEA (7.4) and BA+KIN (7.0). Our findings after the elongation phase, where PGR-free medium performed the best, underscore the importance of a resting or conditioning phase right after cytokinin treatment. In case of carnation species, no study was found where an elongation phase was included in the protocol.

Considering the rooting parameters, in all aspects the best rooting was achieved after BA; it substantially exceeded all other CKs (Table 3). As observed, BA had a superb after-effect on the rooting of the shoots. Therefore, after a 3-week-long treatment with BA (as a source of

cytokinin), then transfer onto PGR-free resting/conditioning medium for a two weeks was applied before placing the shoots on rooting medium. By applying low concentration (0.54 μM) NAA successfully induced rooting on *in vitro* shoot of *D. plumarius*, high rooting percentage (86.7 %) was achieved. For rooting of *in vitro* shoots of related *Dianthus* species, PGR-free medium (Holobiuc and Blindu, 2006; Jarda et al., 2011), or medium supplemented with auxin alone (Holobiuc et al., 2010, 2013) or combined with some type of cytokinins (Cristea et al., 2013) were applied. Rooting rate varied between 30 and 86 % (Pop and Pamfil, 2011; Cristea et al., 2013). However, the after-effect of the CK applied in the shoot multiplication medium was not examined. The best percentage of acclimatization varied between 50 and 80 % in other *Dianthus* species (Cristea et al., 2013; Holobiuc et al., 2013); similarly, in present study 71 % of plantlets acclimatized and developed further.

5. Conclusions

A protocol for the optimal micropropagation of *Dianthus plumarius* subsp. *praecox* was developed (Fig. 2). Culture establishment and maintenance can be done on PGR-free MS medium. Regarding *in vitro* shoot multiplication, the most suitable is the MS medium fortified with 4.5 μM ZEA and 0.54 μM NAA. After achieving the desired number of shoots during the mass propagation phase, conditioning the shoot clusters on a PGR-free medium for 2 weeks is suggested. After this resting phase, individual shoots excised from the clusters need to be transferred onto MS medium containing 4.5 μM BA with 0.54 μM NAA for a 2-week-long period. Rooting should be induced on MS medium supplemented with 0.54 μM NAA. For successful acclimatization (71 %) of plantlets, commercial potting mix and tap water is suitable; the hardening period is 2-week-long.

CRedit authorship contribution statement

Pál Szarvas: Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization. **Dóra Farkas:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Judit Csabai:** Writing – review & editing, Writing – original draft, Formal analysis, Conceptualization. **Angéla Kolesnyk:** Methodology, Conceptualization. **Judit Dobránszki:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

I have shared the link at the Attach File Step

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