ACTA BIOLOGICA CRACOVIENSIA Series Botanica 63/1: 55–61, 2021
DOI: 10.24425/abcsb.2020.131674

MICROPROPAGATION OF FIVE ENDEMIC, RARE AND/OR ENDANGERED *NARCISSUS* SPECIES FROM THE IBERIAN PENINSULA (SPAIN AND PORTUGAL)

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Received October 26, 2020; revision accepted February 17, 2021

The genus *Narcissus* has several endemic, rare and/or threatened species in the Iberian Peninsula and North Africa. *In vitro* propagation is a useful tool for threatened plants conservation used in *ex situ* strategies. Thus, the aim of this work was to study the propagation *in vitro* of bulb scale explants of five endemic, rare and/or endangered *Narcissus* species from the Iberian Peninsula, treated with different PGR combinations. Initiation was achieved in half-strength Murashige and Skoog (MS) basal salts and vitamins, 10 g/L sucrose, 500 mg/L casein hydrolysate, 2 mg/L adenine, 10 mg/L glutathione and 5.5 g/L plant agar. In the multiplication phase, the highest bulblet proliferation was obtained in MS medium supplemented with 30 g/L sucrose and the combination of 10 μM 6-Benzylaminopurine (BAP) + 5 μM α-Naphthaleneacetic acid (NAA) in *N. alcaracensis*, *N. eugeniae* and *N. hedraeanthus*; 20 μM BAP + 5 μM NAA in *N. jonquilla* and *N. yepesii*. The highest rooting was obtained with 5 μM NAA + 1 μM Indole-3-butyric acid (IBA) for all species (>75%) and more than 80% of the produced bulblets were successfully acclimatized.

Key words: bulb scales, conservation, endangered plants, micropropagation, *Narcissus*, plant growth regulators

INTRODUCTION

Narcissus cultivars are very popular for gardening in parks, public spaces, home gardens, as cut flowers or potted flowers, and they also display interesting pharmaceutical applications (Hanks, 2002). In addition, rare *Narcissus* wild taxa are in high demand, and have come out in several sites all over the world (The Daffodil Society, 2019). Several wild species of this genus are also endemic to restricted territories and threatened in the Iberian Peninsula, according to the International Union for the Conservation of Nature (https://www.iucnredlist.org/) and national (Bañares et al., 2010) or regional (DOCM, 1998) checklists. Although micropropagation of *Narcissus* cultivars has been quite extensively studied (Hussey, 1982; Squires et al., 1991; Chow et al., 1992; Harvey and Selby, 1997; Langen-Gerrits and de Klerk, 1999; Sage et al., 1999; Sochacki and Orlikowska, 2005; de Klerk, 2012; Malik and Bach, 2016, 2017), information on micropropagation of wild species is rather

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PL ISSN 1898-0295

limited. Successful *in vitro* production has been achieved for *N. bulbocodium* (Santos et al., 1998), *N. triandrus* (Santos and Salema, 2000), *N. asturiensis* (Santos et al., 2002) and *N. tazetta* (Rahimi Khonakdari et al., 2020). The *in vitro* culture of *N. longispathus* Pugsley, *N. nevadensis* Pugsley and *N. tortifolius* Fdez. Casas. has also been conducted in genebanks (Bañares et al., 2010). However, there are more than 20 rare, endemic and threatened *Narcissus* species listed in the Iberian Peninsula (Spain and Portugal) as well as in North Africa where any information regarding their micropropagation is still lacking. As micropropagation is a useful tool for plant conservation biology that can be applied both in *ex situ* and *in situ* strategies (González-Benito and Martín, 2011), the present study, for the first time, focused on development of micropropagation procedures for five of these *Narcissus* species from the Iberian Peninsula: *N. alcaracensis* S. Ríos, D. Rivera, Alcaraz & Obón; *N. eugeniae* Fdez. Casas, *N. hedraeanthus* (Webb & Heldr.) Colmeiro, *N. jonquilla* L. and *N. yepesii* S. Ríos, D. Rivera, Alcaraz & Obón.

MATERIAL AND METHODS

Mother plants were selected from the *Narcissus* collection based in the Botanical Garden Torretes (Ibi, Spain) and bulbs (collected in April-May) were used as a source of explants. The bulbs were washed in tap water with commercial soap (Domestos, London, UK) containing sodium hypochlorite at 4.5 g 100 g^{-1}. Next, the bulbs were placed in a hot-water treatment consisting in 60 min immersion at 54°C, followed by immersion in 7% (w/v) $Ca(ClO)_2$. Then, uniform explants (about 3-5 mm width and 5-10 mm length) were prepared adapting the twin-scale approach by bulb dissection in pieces containing about 2 mm of the bottom plate. These scales were transferred onto initiation medium IN1 containing full--strength MS, 30 g/L sucrose, 500 mg/L casein hydrolysate, 2 mg/Ladenine, 10 mg/L glutathione and 5.5 g/L plant agar (Duchefa, The Netherlands) or the same composition plus 2 g/L activated charcoal (AC) for the initiation medium IN2. Culture conditions during the initiation stage were as follows: temperature 24±1°C in dark for 10 days. Then, the explants were placed for 30 days under a 16-hours photoperiod of red-blue light (intensity of 42 μmol $m^{-2}s^{-1}$) using T8 Gro-lux tubes (Sylvania Lamps, Germany) at 24°C. In both initiation media, bulb-scale explants were put in 25 × 150 mm culture tubes with polypropylene caps (Auxilab S.L., San Ginés, Spain). Three replicates consisting of 20 explants each were placed under cultivation for the initiation phase during 40 days in total. After this period, contaminated (either by fungi, bacteria or both) or dead explants were discarded and those explants showing signs of growth (bulblet formation) were considered initiated under *in vitro* conditions, counted, and selected for further experiments. Initiated cultures, either in IN1 or IN2, were further transferred into multiplication medium containing full-strength MS, 30 g/L sucrose, 5.5 g/L plant agar and 5 μM BAP + 5 μM NAA for 60 days under the same photoperiod and light intensity as mentioned above. Six passages of cultivation of 60 days each were done in order to obtain a stock of bulblets *in vitro* prior to development of the multiplication experiments. Finally, all bulblets obtained as stock were cultured during 30 days in full-strength MS, 30 g/L sucrose, 5.5 g/L plant agar (without plant growth regulators, PGRs) in order to stabilize the PGRs content of the plants to be employed in multiplication trials. For multiplication experiments, four combinations of PGRs using 6-Benzylaminopurine (BAP), α-Naphthaleneacetic acid (NAA) and Indole-3-butyric acid (IBA) were tested. To assess the bulblet production during the multiplication phase the following combinations were used: PGRs-free medium (M1) and mixtures containing 5 μM BAP + 5 μM NAA (M2), 10 μM BAP + 5 μM NAA (M3) and 20 μM BAP + 5 μM NAA (M4). For multiplication, three replicates consisting of 20 explants each were placed under cultivation in the same photoperiod conditions as those explained for initiation. Newly formed bulblets per explant were recorded after 60 days of cultivation on multiplication medium to perform the rooting trials. For rooting experiments, four rooting media composed of MS basal salts and vitamins, 30 g/L sucrose, 5.5 g/L plant agar, and four combinations of PGRs were employed. The PGR combinations were as follows: a growth regulator--free medium (R1), 5 μM NAA (R2), 5 μM NAA + 1μM Indole-3-butyric acid, IBA (R3) and 5 μM NAA + 1μM indole-3-acetic acid, IAA (R4). As performed in multiplication phase, the rooting experiments were carried out at 24±1°C in a growth chamber under a 16-hours photoperiod of red-blue light (intensity of 42 μmol $m^{-2}s^{-1}$) using T8 Gro-lux tubes (Sylvania Lamps, Germany). Rooting evaluation was visually scored after 30 and 60 days of cultivation on each

rooting medium according to the methodology described for *Lapiedra martinezii* Lag. in Juan-Vicedo et al. (2019): 0 = no roots; 1 = roots <0.5 cm; 2 = 1-2 roots ≥ 1 cm or any number of roots 0.5-1 cm long; 3 = 3 or more roots ≥ 1 cm. After rooting experiments, rooted bulblets showing calibers around 5 mm width and 5–10 mm length, as well as roots longer than 1 cm were selected for acclimatization experiments. These bulblets were previously subjected to cold exposure of 10°C, at 42 μmol $m^{-2}s^{-1}$, for 30 days in order to break dormancy. Then, bulblets were transferred to *ex vitro* conditions by planting rooted bulblets in an autoclaved (30 minutes at 121°C) soil mixture of peat moss and vermiculite (5:4 ratio) (Batlle, Molins de Rei, Spain) for 60 days during a double-phase experiment. In the first phase (acclimatization), pots were placed in a growth chamber with light and humidity control at a constant temperature of 24±1°C, 100% relative humidity (RH), and a 16-hours photoperiod using red-blue light (T8 Gro-lux tubes, Sylvania Lamps, Germany, with photosynthetically active radiation of 42 μmol $m^{-2}s^{-1}$) during 30 days. In the second phase (hardening), the RH was decreased to 70% and light intensity was increased up to 80 μmol $m^{-2}s^{-1}$ for 30 days. At the end of the experiment, the pots were placed in shade in outdoor conditions for 40 days and the survival was recorded. All experiments (initiation, multiplication, rooting and acclimatization) were performed using three replicates of 20 explants each and means as well as standard errors were calculated in order to perform statistical tests. A summary of the experiments is presented in Table 1: all set experiments were done by triplicate. For the data analyses, one-way analyses of variance (ANOVA) were conducted and significant differences between means were obtained with the Tukey's honestly significant difference (HDS) test at the 5% level. All data were analyzed using the Infostat 2008 package (Di Rienzo et al., 2008). Percentages of rooting and acclimatization were previously arcsine square root transformed whereas the bulblet formation was analyzed without any transformation.

RESULTS AND DISCUSSION

Initiation of bulb-scale explants was more successful in initiation media not containing AC (IN1) for all species tested (Table 2). The use of AC on plant *in vitro* cultures has been related to its role in

TABLE 1. Summary of the experiments performed for bulb-scale micropropagation of *N. alcaracensis*, *N. eugeniae*, *N. hedraeanthus*, *N. jonquilla* and *N. yepesii*.

Stage	**Explant (number)**	**Media**	**Culture conditions**	**Culture period (days)**
Initiation *in vitro*	100-183	IN1, IN2	**Temperature:** 24±1ºC **Light:** dark for (10 days) and 16-hours photoperiod of 42 μmol $m^{-2}s^{-1}$ (30 days)	40
Multiplication *in vitro*	60 (3 replicates of 20)	M1, M2, M3, M4	**Temperature:** 24±1ºC **Light:** 16-hours photoperiod of 42 μmol $m^{-2}s^{-1}$	30
Rooting *in vitro*	60 (3 replicates of 20)	R1, R2, R3, R4	**Temperature:** 24±1ºC **Light:** 16-hours photoperiod of 42 μmol $m^{-2}s^{-1}$	60
Cold exposure	60 (3 replicates of 20)	R1, R2, R3, R4	**Temperature:** 10ºC **Light** 16-hours photoperiod of 42 μmol $m^{-2}s^{-1}$	30
Acclimatization *ex vitro* I	60 (3 replicates of 20)	Peat moss: vermiculite (5:4)	**Temperature:** 24±1°C **Relative Humidity:** 100% **Light:** 16-hours photoperiod of 42 μmol $m^{-2}s^{-1}$	30
Acclimatization *ex vitro* II (Hardening)	60 (3 replicates of 20)	Peat moss: vermiculite (5:4)	**Temperature:** 24±1°C **Relative Humidity:** 70% **Light:** 16-hours photoperiod of 80 μmol $m^{-2}s^{-1}$	30

Media: IN1 (full-strength MS, 30 g/L sucrose, 500 mg/L casein hydrolysate, 2 mg/L adenine, 10 mg/L glutathione and 5.5 g/L plant agar), IN2 (IN1 plus 2 g/L AC), M1 (growth regulators free), M2 (5 μM BAP + 5 μM NAA), M3 (10 μM BAP + 5 μM NAA), M4 (20 μM BAP + 5 μM NAA), R1 (growth regulators free), R2 (5 μM NAA), R3 (5 μM NAA + 1 μM IBA) and R4 (5 μM NAA + 1 μM IAA).

TABLE 2. Effect of two initation media on the initiation success of bulb-scale explants of *N. alcaracensis*, *N. eugeniae*, *N. hedraeanthus*, *N. jonquilla* and *N. yepesii*. Results are expressed as the number of explants cultured, contaminated (either by bacteria, fungi or both), dead and recovered/healthy explants that restored growth within 40 days of culture.

Species	Treatment	Cultured explants	Contaminated explants	Dead explants	Recovered/ Healthy explants
	IN1	119	75	27	17
N. alcaracensis	IN2	100	80	15	5
	IN1	158	63	67	28
N. eugeniae	IN2	161	69	74	18
	IN1	183	73	63	47
N. hedraeanthus	IN2	180	75	78	27
	IN1	161	80	47	34
N. jonquilla	IN2	158	89	58	11
	IN1	107	52	43	12
N. yepesii	IN2	105	51	47	7

Treatments: IN1 (full-strength MS, 30 g/L sucrose, 500 mg/L casein hydrolysate, 2 mg/L adenine, 10 mg/L glutathione and 5.5 g/L plant agar), IN2 (IN1 plus 2 g/L AC).

adsorption of substances (e.g., phenolics and their oxidates, inhibitory compounds) that can be harmful for the new tissues formed *in vitro* (Thomas, 2008). In bulbous plants' multiplication *in vitro*, AC was related to a higher bulb size in *Lilium* sp. (Bachetta et al., 2003) and improved shoot regeneration in *L. longiflorum* explants (Nhut et al., 2001). AC also promoted bulblet production both in already initiated cultures of *N. tazetta* (Steinitz and Yahel, 1982) and in *N. tazetta* "Chinensis" (Abdel-Rahman et al., 2017). However, other compounds that can be adsorbed by AC are organic supplements and growth regulators (Thomas, 2008). Due to the importance of these compounds to restore the plant growth at the initiation of the *in vitro* cultures (Langen-Gerrits and de Klerk, 1999; Iliev et al., 2010), it seems reasonable to think that AC could sometimes impede the intake of these substances and, in turn, give lower response at the initiation stage as reported here. In fact, AC in culture medium was also related to a decrease in bulblet differentiation in *Lilium* (Takayama and Misawa, 1980) and in initiation success also in *L. martinezii* (Juan-Vicedo et al., 2019). After the cultures had been initiated, multiplication experiments were performed. It is usually reported that reduced NAA concentrations in comparison to BAP promote *in vitro* bulblet proliferation in geophytes (Hussey, 1982; Seabrook, 1990; Santos et al., 1998; Langen-Gerrits and de Klerk, 1999; Santos et al., 2002; Freytag et al., 2017; Juan-Vicedo et al., 2019; Rahimi Khonakdari et al., 2020), as revealed in this study. However, determination of the specific concentration of these PGRs in culture media is crucial for successful micropropagation of the target species (de Klerk, 2012; Freytag et al., 2017). Among the four combinations of PGRs used for multiplication in the present work, the best response was obtained in M3 (10 μM BAP + 5 μM NAA) that showed significantly higher bulblet induction in *N. alcaracensis* (4.35±0.59), *N. eugeniae* (5.95±0.60) and *N. hedraeanthus* (9.00±0.72). Also, M4 (20 μM BAP + 5 μM NAA) promoted the highest bulblet formation in *N. jonquilla* (7.40±1.19) and *N. yepesii* (4.05±0.69) (Table 3). The combinations M1 (growth regulators free) and M2 (5 μM BAP + 5 μM NAA) yielded much lower than M3 and M4 in all studied species. Taking into account each species' multiplication yield, the highest bulblet proliferation was obtained in *N. hedraeanthus* (9 bulblets per explant). This is in agreement with the yield obtained by Santos et al. (1998) for the closely related species *N. bulbocodium* L. (*Narcissus* L. Section *Bulbocodii* DC). *N. jonquilla* performed a lower response (7.40 bulblets per explant). To the best of our knowledge, this is the first report

TABLE 3. Effect of four combinations of growth regulators on bulb-scale explant multiplication of *N. alcaracensis*, *N. eugeniae*, *N. hedraeanthus*, *N. jonquilla* and *N. yepesii*. Mean values per species within a column followed by different letters represent significant differences between treatments according to Tukey's HSD test ($P<0.05$).

Species	Treatment	Number of bulblets
	M1	0.65±0.49c
N. alcaracensis	M2	1.00±0.46c
	M3	4.35±0.59a
	M4	2.65±0.67b
	M1	0.70±0.0.47c
N. eugeniae	M2	2.15±0.37b
	M3	5.95±0.60a
	M4	2.05±0.69b
	M1	0.60±0.50d
N. hedraeanthus	M2	1.15±0.37c
	M3	9.00±0.72a
	M4	4.00±0.65b
	M1	1.90±0.79d
N. jonquilla	M2	3.75±1.02c
	M3	5.95±0.51b
	M4	7.40±1.19a
	M1	1.10±0.31c
N. yepesii	M2	1.55±0.76bc
	M3	2.05±0.69b
	M4	4.05±0.69a

Treatments: M1 (growth regulators free), M2 (5 μM BAP + 5 μM NAA), M3 (10 μM BAP + 5 μM NAA) and M4 (20 μM BAP + 5 μM NAA).

concerning micropropagation of wild jonquils (*Narcissus* L. Section Jonquillae DC). Chow et al. (1992) found an increase in bulblet proliferation from shoot cultures in *N. jonquilla* after transference of shoot clumps from 3% to 9% sucrose. However, there was no information on direct bulblet formation from bulb explants using PGRs and therefore we cannot establish whether multiplication performance is in accordance with other species of the Section. In any case, the yield obtained in this study is still high taking into account the results of the five studied *Narcissus* species. In contrast, *N. eugeniae*, *N. yepesii* and *N. alcaracensis* gave the lowest yield (4.05–5.95 bulblets per explant). These results may suggest that the range of combinations of PGRs used in this study might be not optimal for bulb multiplication in these taxa. However, Santos et al. (2002) also obtained similar optimal micropropagation yields in the closely related species *N. asturiensis* (Jord.) Pugsley, which could indicate that wild species belonging to the *Narcissus* Section Pseudonarcissi DC (trumpet daffodils) might have lower bulblet production *in vitro* in comparison to other members of the genus. However, further research would be desirable to check whether there exists a different multiplication performance depending on the taxonomical group where they belong and in turn, to optimize the bulblet proliferation *in vitro*. It is generally accepted that bulbs do not need rooting treatments (Langen-Gerrits and de Klerk, 1999) as they can spontaneously achieve high rooting percentages in multiplication medium (Juan-Vicedo et al., 2019). In *Narcissus* bulblets, rooting treatment is normally necessary to achieve higher sprouting rates in micropropagated bulbs after transference to *ex vitro* conditions (de Klerk, 2012) and, therefore, to ensure a successful micropropagation protocol. In our experiments, variable percentages of root induction were obtained in all tested combinations of PGRs (including the controls, R1) for the five species studied during the 30-days rooting period. When the cultivation period was extended up to 60 days all these percentages significantly increased and the rooting medium R3 containing 5 μM NAA + 1μM IBA performed the best response (2–3 roots showing about 1 cm length) in the five studied species (Table 4). Finally, acclimatization success was higher than 80% for all rooted bulblets (Table 4). In this work, a satisfactory specific method of micropropagation for five endemic, rare and/or threatened *Narcissus* was described for the first time. These results provide relevant information for the conservation of these species *ex situ*. In addition, given the great importance of *Narcissus* species in the galanthamine production (Hanks, 2002; Rahimi Khonakdari et al., 2020) we expect that the results presented in this work could be helpful for further studies on alkaloid biosynthesis using *in vitro* systems.

AUTHORS' CONTRIBUTION

JJV performed the experiments, analysis and interpretation of data, as well as drafted the manuscript. AP helped in the work design and in manuscript drafting. SR carried out the field work and developed the idea and JLCM participated in the experiment designing and manuscript drafting. The authors declare that there is no conflict of interest.

TABLE 4. Effect of four combinations of growth regulators and two periods of culture on rooting of regenerated bulblets as well as acclimatization success of rooted bulblets for *N. alcaracensis*, *N. eugeniae*, *N. hedraeanthus*, *N. jonquilla* and *N. yepesii*. Mean values per species within a column followed by different letters represent significant differences between treatments according to Tukey's HSD test ($P<0.05$).

Species	Treatment	Rooting (% at 4 weeks)	Rooting score (4 weeks)	Rooting (% at 8 weeks)	Rooting score (8 weeks)	Acclimatization (%)
	R1	30.25d	0.09	62.75c	1.88	90a
N. alcaracensis	R2	42.50c	1.3	79.00b	2.37	95a
	R3	74.75a	2.25	92.37a	2.77	100a
	R4	51.75b	1.5	79.00b	26.07	90a
	R1	37.00c	1.1	58.00b	1.74	80a
N. eugeniae	R2	38.25c	1.15	61.75bc	1.85	80a
	R3	78.50a	2.35	93.50a	2.8	95a
	R4	59.50b	1.8	64.75b	1.94	90a
	R1	35.5c	1.06	40.25b	1.2	95a
N. hedraeanthus	R2	48.25a	1.5	65.00b	1.95	100a
	R3	47.75a	1.4	94.25a	2.8	95a
	R4	40.00b	1.2	57.25c	1.7	90a
	R1	33.75b	1.0	57.75c	1.73	80a
N. jonquilla	R2	37.75b	1.13	63.75b	1.9	80a
	R3	44.75a	1.34	81.00a	2.43	80a
	R4	36.50b	1.09	54.25c	1.63	80a
	R1	25.00d	0.75	47.50c	1.4	90a
N. yepesii	R2	33.25c	1.0	38.50d	1.15	85a
	R3	59.75a	1.8	75.50a	2.26	95a
	R4	48.00b	1.44	64.75b	1.94	95a

Treatments: R1 (growth regulators free), R2 (5 μM NAA), R3 (5 μM NAA + 1μM IBA) and R4 (5 μM NAA + 1μM IAA).

ACKNOWLEDGEMENTS

The authors would like to acknowledge Universitat d'Alacant for the possibility of conducting this work by means of a grant included in the program "Becas y Contratos para la formación de Doctores del Vicerrectorado de Investigación, FPU2010" and specific funding for a research stay to start some of the cultures. Also, this work was partly supported by the R+D+I funding lines of the Spanish Ministry of Environment "Ministerio de Medio Ambiente y Medio Rural y Marino" through the project 126/SGTB/2007/4.1 'Caracterización, conservación y multiplicación de narcisos endémicos de Castilla-La Mancha (gen. Narcissus, Fam. Amaryllidaceae): revalorización de un recurso vegetal para el desarrollo sostenible de la Reserva de la Biosfera La Mancha Húmeda'. Finally, we would also like to thank Tatiana Gocheva and Andrey Marchev (Institute of Microbiology-Bulgarian Academy of Sciences) for their assistance in laboratory and Diana Smith for her linguistic assistance in the final draft of the manuscript.

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