Original paper

Ex situ preservation in medium-term culture of the threatened taxon Dianthus nardiformis Janka

IRINA HOLOBIUC¹, RODICA CATANĂ¹, FLORENȚA HELEPCIUC¹, CARMEN MAXIMILIAN¹, MONICA MITOI¹, GINA COGĂLNICEANU¹

¹Institute of Biology, Romanian Academy, Bucharest, Romania

Abstract

Our aim was to elaborate an efficient and reproducible protocol for medium-term culture of the threatened taxon Dianthus nardiformis. To reduce the growth, sucrose, mannitol, polyethylene glycol, Abscisic acid and Jasmonic acid were tested. For assessing the in vitro response, the growth and regeneration were registered after different time intervals.

Mannitol is the most effective for medium-term preservation viable cultures which can be maintained unlimited time through transfer at every 3 months. In its presence, somatic embryogenesis was induced and in vitro growth in the minimal cultures was reduced between 9 and 12 times comparing to the control.

Antioxidant enzymes assay revealed qualitative and quantitative differences among the experimental variants, and also between different concentrations of the same compound in correlation with the growth reduction and regeneration. POX was the most suitable to detect the efficiency of different treatments to induce medium-term cultures.

Keywords Medium-term, mannitol, somatic embryogenesis, antioxidant enzymes.

To cite this article: HOLOBIUC I, CATANĂ R, HELEPCIUC F, MAXIMILIAN C, MITOI M, COGĂLNICEANU G. Ex situ preservation in medium-term culture of the threatened taxon Dianthus nardiformis Janka. Rom Biotechnol Lett. 2021; 26(2): 2416-2422. DOI: 10.25083/rbl/26.2/2416.2422
**Introduction**

Plant conservation became an important aim of the humankind, more than one third of plant species being rare, endangered, and threatened by extinction (RAJASEKHARAN & SAHIJRAM, 2015) needing to be preserved both *in situ* and *ex situ*, with 20% available to be reintroduced into the wild.

*Ex situ* conservation based on biotechnology was developed to save wild threatened species (GONZALEZ-BENITO & MARTIN, 2011; KRISHNAN et al, 2011; MUÑOZ-CONCHA & DAVEY 2011, PIJUT et al, 2011; CHAUHAN, 2016). Small amounts of tissues, organs or seeds can be collected without affecting the natural populations (PENCE, 2002). Among different *ex situ* approaches, medium-term preservation is an efficient and not expensive approach (CRUZ et al, 2013), involving growth limiting factors (physical or/and chemical) for reducing the growth to establish minimal or slow growth cultures (CHA-UM & KIRDMANNEE, 2007). Some medium-term preservation protocols were previously developed in other threatened *Dianthus* taxa as *D. callizonus*, *D. tenuifolius*, *D. superbus* L. ssp. *speciosus* (HOLOBIUC & BLINDU 2006), *D. spiculifolius*, *D. glacialis* ssp. *gelidus* (HOLOBIUC et al, 2009b, 2010b), *D. trifasciculatus* (HOLOBIUC et al, 2014), *D. ingoldbyi* (ARDA et al, 2016).

*Dianthus nardiformis* Janka, a perennial plant, with pink flowers, is an endemic endangered vulnerable element from the South-East of Romania, Dobrogea region (DIHORU & NEGREAN, 2009) and Bulgaria on the Black-Sea Coast and Danubian Plain (TZONEV 2004).

The aim of our study was to establish a medium-term culture protocol using growth limiting compounds for *ex situ* preservation of this taxon. The effect of different factors was evaluated by measuring the growth and regeneration in correlation with the effect of oxidative stress effect on antioxidant enzymes.

**Material and Methods**

*Plant material and culture conditions*: double node stem fragment of about 10 mm detached from vitroplants (HOLOBIUC et al, 2009) were used as explants. Five inocula were cultured on a jar of 6 cm diameter/10 cm high, with 5 jars/variant (25 replicates/variant). All the cultures were maintained in the growth chamber at 2000 lux (27 μmol/m²/s), with 16/8 photoperiod and 25°C temperature. The media variants tested to induce growth retardation were based on MS formula (MURASHIGE & SKOOG, 1972), added with B5 vitamins mixture (GAMBORG, 1968), solidified with agar (Duchefa Plant Agar) 8 g/l and pH adjusted at 5.8 before autoclavage. MS medium was supplemented with different compounds: M1-87.6 mM sucrose used as control, M2-87.6 mM sucrose +164.7 mM mannitol, M3-87.6 mM sucrose +329 mM mannitol, M4-175.2 mM sucrose, M5-262.9 mM sucrose, M6-87.6 mM sucrose + 60 mM PEG 4000, M7-87.6 mM sucrose + 75 mM Abscisic acid (ABA), M8-87.6 mM sucrose +23 mM Jasmonic acid (JA).

**Results**

*Evaluation of medium-term cultures*: On the control variant, the lateral shoots developed well and fast, several regenerants emerged from the lateral meristems and rooting occurred easily. In the case of PEG, mannitol at lower level and sucrose addition, despite the induced osmotic stress, lateral shoots developed from the first weeks (Fig. 1).
After 40 days of culture, a decreasing of the tallest regenerant length on the variants supplemented with osmolytes as sucrose, mannitol and PEG (M2, M3, M4, M5, M6) was recorded (Table 1). PEG addition determined a reduction of growth (Fig. 1a), but its effect was lower comparing to mannitol (Fig. 1b).

**Table 1.** Evaluation of medium-term cultures of *D. nardiformis.* The values represent the average ± SD, the results with the same letter did not differ significantly.

| Variant | Maximum regenerants length/ explant (mm) | The number of regenerants/ explants |
|---------|----------------------------------------|-----------------------------------|
|         | 40 days                                | 80 days                           | 120 days                          |
| M1      | 22.4±12.77a                            | 50.26±29.72a                      | 7.26±3.33a                       |
| M2      | 2.4±0.50b                             | 9.0±1.0.03c                       | 8.6±8.71a                       |
| M3      | 1.8±0.51b                             | 2.8±1.20b                        | 2.6±0.82b                       |
| M4      | 13.4±7.44a                            | 30.2±23.60a                      | 4.3±1.67b                       |
| M5      | 4.2±1.66c                             | 10.8±14.29c                      | 4.6±2.06b                       |
| M6      | 4.0±1.02c                             | 6.2±1.61c                       | 5.6±3.99b                       |
| M7      | 2.4±1.80b                             | 3.4±1.46c                       | 1.13±0.3 b                      |
| M8      | 19.3±19.33a                           | 31.9±19.00a                     | 7.0±5.50a                       |

Mannitol at 329 mM level (M3) was the most limiting factor, with small buds emerging from the lateral meristems (Fig. 1b). In the variant M2, with lower mannitol level (164.7 mM), the effect was not so strong, but the regenerants length was reduced comparing to the control. Presence of 175.2 mM sucrose (M4) did not significantly reduce the growth of the shoots, but the higher level (262.9 mM-M5) had a more pronounced effect (Fig. 1c, Table 1) similarly to PEG added variant (M6). ABA (75 mM in M7) inhibited the growth of the meristems, they remaining viable for 3-4 months, no rooting occurred. JA (23 mM-in M8 variant) did not affect the growth and the number of shoots. The extension of culture at 80 days, ensured the growth limitation in the case of mannitol, PEG and sucrose (Table 1). While roots did not develop in presence of PEG, it grown on mannitol media.

**Figure 1.** Aspects of medium-term cultures of *D. nardiformis* on MS medium added with: a) 60 mM PEG; b) 329 mM mannitol; c) 262.9 mM sucrose after 40 days of culture.

**Figure 2.** Aspects of medium-term cultures of *D. nardiformis* maintained on 164.7 mM mannitol-added medium (M2): during 60 days (a), 80 days (b), detail of a highly regenerative aggregate at 80 days (c), 120 days (d) and respectively on medium added with 262.9 mM sucrose (M5) 80 days (e), 329 mM mannitol (M3) during 120 days (f) and 60 mM PEG (M6) 160 days with vitrification (g).
The regenerative structures after 40 days (Table 1) varied between 1.13 in presence of ABA and 8.66 on mannitol lower level. After 80 days, the best results of regeneration were recorded on M2 at 164.7 mM mannitol (Fig. 2b, c) and M6 – medium with PEG (Fig. 2g, Table 1). Sucrose at high level determined the etiolation and death of already developed shoots, despite some new buds emerged, they degenerated over time (Fig. 2e). On the variant with JA (M8), the development is the same as in control, continued to grow and rooting occurred easily. After 120 days, despite the limited growth, the regeneration increased in the case of M2, M3, M6 variants (Table 1). Despite the good regeneration in presence of PEG, vitrification was noticed.

Figure 3. Aspects of direct somatic embryogenesis in D. nardiformis induced in the presence of mannitol after 120 days of culture in fresh samples: (a) Globular somatic embryos; (b) Developed plantlet originated in somatic embryos; (c, d). Histological sections of embryogenic aggregates – different stages of SE.

Biochemical analysis in D. nardiformis medium-term cultures

POX spectrum revealed qualitative and quantitative differences among the experimental variants, and also between different concentrations of the same compound (Fig. 4D), being correlated very well with the growth. In case of limited growth, the bands from the fast migration zone disappeared as in the case of the high level of mannitol (M3) 5, 6 and 7 isoforms or had a low expression for M5 variant with increased sucrose and for M6 with PEG. The POX activity is highly correlated with its spectrum, a decrease was detected in the case of high levels of osmolytes (M3, M5, M6) (Fig. 4A), while variants with moderate levels (M2, M4) had an increased activity. The SOD activity decreased in the case of higher levels of osmolytes as in M3 (329 mM mannitol), M5 (262.9 mM sucrose), M6 (60 mM PEG) being also correlated with the inhibition of the elongation process and growth (Fig. 4B). Regarding SOD spectrum, three isoforms were expressed in all variants, but one band was more intense on 329 mM mannitol (M3) (Figure 4E).

In case of CAT, the electrophoresis revealed differences in enzyme banding pattern for mannitol and PEG variants (Fig. 4F). It can be noticed a slightly decrease of the CAT activity (Fig. 4C) on higher levels of mannitol and sucrose supplement variants.

Discussions

The survival and regeneration of in vitro culture is affected by chemical factors used to induce minimal cultures, imposing to establish the most suitable approach. While in vitro response and growth of D. nardiformis was good (HOLOBIUC et al., 2009a, 2010a), the reaction in restricted growth conditions was different depending on compound and level applied: axillar shooting (PEG, sucrose, JA, mannitol at lower level), SE in the case of mannitol at higher concentration and after the prolonged exposure to lower level similar to other experiments in Dianthus related taxa (HOLOBIUC et al., 2009b, 2010b, 2014).
Mannitol proved the most suitable to establish medium-term culture, while in case of PEG, despite the growth limitation and good regeneration, during the time, vitrification appeared. Sucrose reduced the growth and ensured the regeneration, but at the lower level its effect is no so pronounced, and at higher level despite a limited growth, etiolation appeared because the stress. ABA did not allowed in vitro development at level tested, just keep alive lateral meristems of the explants for a limited period. ABA was reported to induced growth inhibition, improving the drought and salt resistance, besides its role in senescence (SHARP et al, 1994). JA in our experiment did not affect the growth and shoots formation which were as in control, despite it was reported to be a growth inhibitor (KIM et al, 2009). SE reported in D. nardiformis was similarly as in other Dianthus sp. (HOLOBIUC et al, 2009c) allowing a high regeneration, being a source propagules for conservative and practical aim. Direct SE from leaf with adition of growth regulators was reported in a cultivated Dianthus, PEG being necessary just for embryos maturation in short-term culture (YANTCHEVA et al, 1998). In our experiment, mannitol was a factor of growth limitation, but also a promotor of SE. The are evidences that embryogenic competence is activate by osmolytes (FEHER et al, 2003). Different stress factors was also reported to induce SE (ZAVATTIERI et al. 2010) as cold, heat, osmotic or nutrient stress probably because they stimulate the endogenous synthesis of ABA at a level which can trigger the somatic embryogenesis (KARAMI & SAIDI, 2008). In a related taxon D. ingolbyi, a reliable medium-term protocol at 4°C with 58% survival rate was reported (ARDA et al, 2016), but mannitol was better tolerated, with a significant growth reduction between 9-12 times comparing to the control, correlated with 90-100% survival and with a high regeneration. Concerning biochemical evaluation, POX, SOD and CAT spectrum were correlated with variants tested as in a former study in Dianthus sp., antioxidant enzymes being susceptible to the nature, level, duration of exposure to osmolytes and by the capacity of the species to overcome the stress (MITOI et
al, 2009). The three POX isoforms might be involved in cell elongation, exhibiting the highest polymorphism. These enzymes counteract ROS (hydroxyl anion and hydrogen peroxide) which can induce cell damages and even death if protective mechanism fails to detoxify (POLLE, 1995). On the other hand, mannitol can be accumulate in significant amounts, lowering cellular osmotic potential acting also as ROS scavenger (BOHNERT & SHEVELEVA, 1998, RUIJTER et al, 2003). Absence of POX isoforms on higher mannitol may be due to mannitol ROS scavenger role. For SOD, the isoforms were expressed in all variants, also with one band more intense at higher mannitol variant. CAT spectrum revealed differences on mannitol and PEG variants where regeneration was higher. Lower level of osmolytes allowed to plants to overcome the oxidative stress through increasing the antioxidant enzymes activity, but at higher stress, the plants capacity to neutralize ROS declines, some phenotypical changes could appear (dwarfness, etiolation), the viability loss, death or activation of survival mechanisms (as SE). Similary, in D. trifasciculatus, biochemical analyses also shown that POX and CAT were sensitive to the different treatments, increased mannitol influenced the intensity of the bands even two months after its removal (HOLOBIUC et al, 2014).

Conclusions

The factors tested for minimal cultures had different effects on the limitation of the growth and development of regeneratives structures. Mannitol was the most efficiently to ensure a growth limitation of 9 to 12 times lower comparing to the control and to induce as a stress response, a high regeneration through SE generating hundred of propagules in small space and with reduced labor. Biochemical analysis after 120 days proved that antioxidant enzymes activity increased in a compensated system that tend to overcome the oxidative stress generated by different chemical factors while in the decompensated system, antioxidant enzymes activity was decreased, being not able to counteract the oxidative stress. POX was the most sensitive to detect the effect of factors used to limit in vitro growth. Isoenzymes patterns were in fact correlated to the adaptation to osmotic stress, growth reduction and regeneration process.

Acknowledgements

This study was funded by the projects “Biodiversity consolidation through ex situ preservation and somaclonal variability evaluation using molecular techniques in endemic or endangered taxa from Natura 2000 Romanian sites” (31-008/18.09.2007) and “Plant biotechnology for biodiversity conservation and durable development” RO1567-IBB06/2017.

Conflict of Interest

The authors have no conflict of interest to declare.

References

1. ARDA H, DAYAN S, KARTAL C, GÜLER N. In vitro conservation of critically endangered Dianthus ingoldbyi Turrill under slow growth conditions. Trakya University Journal of Natural Sciences. 2016; 17(1): 47-54.
2. BOHNERT HJ, SHEVELEVA E. Plant stress adaptations-making metabolism move. Curr Opin Plant Biol. 1998; 1:267-274.
3. BEAUCHAMP C. and FRIDOVICI I, 1971. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal. Biochem. 1971; 44: 276-287.
4. CHAUHAN RS. Biotechnological approaches for conservation of rare, endangered and threatened plants. Int Journal of Scientific and Research Publications. 2016; 6(12): 10-14.
5. CHA-UM S, KIRDMANEE C. Minimal Growth in vitro culture for preservation of plant species. Fruit, Vegetable and Cereal Science and Biotechnology. 2007; 1(1): 13-25.
6. CRUZ-CRUZ CA, GONZÁLEZ-ARNA OMT, ENGELMANN F. Biotechnology and conservation of plant biodiversity. Resources. 2013; 2: 73-95.
7. DIHORU GH, NEGREAN G. Cartea roşie a plantelor vasculare din România. Editura Academiei Române, Bucureşti. 2009.
8. FEHER A, PASTERNAK TP, DUDITS D. Transition of somatic plant cells to an embryogenic state. Plant Cell Tissue Organ Cult. 2003; 74: 201-228.
9. GAMBORG OL, MILLER RA, OJIMA K. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res. 1968; 50: 151-158.
10. GARCIA-LIMONES C, HERVÁS A, NAVASCORTÉS JA, JIMÉNEZ-DÍAZ RM, TENA M. Induction of an antioxidant enzyme system and other oxidative stress markers associated with compatible and incompatible interactions between chickpea (Cicer arietinum L.) and Fusarium oxysporum f. sp. ciceris. Physiol Mol Plant Pathol. 2002; 61(6): 325-337.
11. GONZÁLEZ-BENITO ME, MARTIN C. In Vitro preservation of spanish biodiversity. In Vitro Cell. Dev. Biol.-Plant. 2011; doi:10.1007/s11627-010-9333-4.
12. HOLOBIUC I, BLÍNDU R. Improvement of the micropropagation and in vitro medium-term preservation of some rare Dianthus species, Contributii Botanice. 2006; 41(2): 143-151.
13. HOLOBIUC I, BLĂNDU R, CRISTEA V. Researches concerning in vitro conservation of the rare plant species Dianthus nardiformis Janka. Biotechnol. & Biotechnol. Eq. 2009 a; 23(2): 221-224.

14. HOLOBIUC M, BLĂNDU R, MITOI M, HELEPCIUC F, CRISTEA V. The establishment of an in vitro gene bank in Dianthus spiculifolius Schur. and D. glacialis ssp. gelidus (Schott Nym. et Kotschy). The initiation of a tissues collection and the characterization of the cultures in minimal growth conditions. Ann For Res. 2009 b; 52: 14-26.

15. HOLOBIUC I, BREZEAU ANA A, BLĂNDU R. Somatic embryogenesis induction in presence of moderate osmotic stress, synthetic seeds production in rare Dianthus species from Romanian Flora as a tool for ex situ conservation. Proc. of the 2nd International Symposium “New Research in Biotechnology” Series F. Special Volume, 2009 Biotechnology, Bucharest, ISSN 1224-7774, 2009c: 69-77.

16. HOLOBIUC I, BLĂNDU R, CRISTEA V. Researches concerning in vitro cultures optimization of the vulnerable species Dianthus nardiformis Janka. Analele Universităţii din Oradea-Fascicula Biologie. 2010a; XVII (1): 116-121.

17. HOLOBIUC I, MITOI M, BLĂNDU R HELEPCIUC F. The establishment of an in vitro gene bank in Dianthus spiculifolius Schur. and D. glacialis ssp. gelidus (Schott Nym. et Kotschy) Tutin: II. Medium-term cultures characterization in minimal growth conditions. Rom. Biotechn. Lett. 2010 b 15 (2):5111-5119.

18. HOLOBIUC I, VOICHIŢĂ C, CATANĂ R, MITOI M, HELEPCIUC F. Medium-term preservation of Dianthus trifascicularis kit ssp. parviflorus through minimal cultures. Muzeul Olteniei Craiova. Oltenia. Studii și comunicări. Științele Naturii. 2014; 30(1): 57-66.

19. KARAMI O, SAIDI A. The molecular basis of stress-induced somatic embryogenesises of Daucus carota. Plant Cell Rep. 2008; 20: 408-415. DOI 10.1007/s11033-009-9764-3

20. KIM EH, KIM YS, PARK SH, KOO YJ, CHOI YD, CHUNG YY, LEE JJ, KIM JK. Methyl Jasmonate reduces grain yield by mediating stress signals to alter spikelet development in rice. Plant Physiolog. 2009; 149: 1751-1760.

21. KRISHNAN P, DECRUSE S, RADHA R. Conservation of medicinal plants of Western Ghats, India and its sustainable utilization through in vitro technology. In Vitro Cell Dev-Pl. 2011; 47. 110-122. doi:10.1007/s11627-011-9344-9.

22. MITOI ME, HOLOBIUC I, BLĂNDU R. The effect of mannitol on antioxidative enzymes in vitro long term cultures of Dianthus tenuifolius and Dianthus spiculifolius, Romanian Journal of Biology - Plant Biology. 2009; 54(1): 25-33.

23. MUÑOZ-CONCHA D, DAVEY M. Micropagation of the endangered Chilean tree, Gomortega keule. In Vitro Cell Dev- Pl. 2011; 47. 170-175. 10.1007/s11627-010-9331-6.

24. PENCE V. Cryopreservation and in vitro methods for ex situ conservation of pteridophytes. Fern Gazette. 2002; 16: 362-368.

25. PIJUT PM, LAWSON SS, MICHLER CH. Biotechnological efforts for preserving and enhancing temperate hardwood tree biodiversity, health, and productivity. In Vitro Cell Dev-Pl. 2011; doi:10.1007/s11627-010-9332-5

26. POLLE A. Mehler reaction- Friend or foe in photosynthesis. Bot. Acta. 1995; 109: 84-94.

27. RAJASEKHARAN PE, SAHIJRAM L. In vitro conservation of plant germplasm. In: Bir Bahadur, Manchikatla Venkat RaAm, Leela Sahijram, K.V. Krishnamurthy (Ed.): Plant Biology and Biotechnology (Vol II: Plant Genomics and Biotechnology). New York: Springer Verlag, 2015; pp. 417-443.

28. RUIJTER GJG, BAX M, PATEL H, FLITTER SJ, VAN DE VONDERVOORT PIJ, RONALD P, DE VRIES R, VANKUYK PA, VISSER J. Mannitol is required for stress tolerance in Aspergillus niger conidiospores. Eukaryot Cell. 2003; (24): 690-698.

29. SHARP RE, WU Y, VOETBERG GS, SAAB I, LENOBLE ME. Confirmation that abscisic acid accumulation is required for maize primary root elongation at low water potentials. J of Exp Bot. 1994; 45: 1743-1751.

30. TZONEV R. New data and summarized information on the chorology of some rare, threatened and endemic plants in the midle Danube plain and Balkan foothill region. Ann. Univ. Sofia “St. Kl. Ohridski” Fac. Biol. 2 – botany. 2004; 97: 59-70.

31. YANCHEVA A, VLADOVA A, KREKOV B, CONCHA D, DAVEY M. Cryopreservation and in vitro methods for ex situ conservation of pteridophytes. Fern Gazette. 2002; 16: 362-368.

32. ZAVATTIERI MA, FREDDERO AM, LIMA M, RUI S, ARNHOLDT-SCHMITT B. Induction of somatic embryogenesis as an example of stress-related plant reactions. Electron J Biotechn. 2010; 13(1): 1-9.