RESEARCHES CONCERNING *IN VITRO* CULTURES OPTIMIZATION OF THE VULNERABLE SPECIES *Dianthus nardiformis* Janka

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Abstract. Our aim was to establish an optimized protocol for *in vitro* short-term conservation of the vulnerable species *Dianthus nardiformis* Janka belonging to Romanian Flora.

For our purpose, different culture media compositions were tested: complex variants added with plant growth regulators and simplified media with low levels of macro- and microelements.

This species had a good *in vitro* reaction, the regeneration occurs through direct morphogenesis as in all *Dianthus* species. Generally, the use of variants of MS medium supplemented with cytokinin/ auxin at 10/1 ratio proved to induce high regeneration response starting from the first month of culture.

In our study, MS media complex variants with diminished levels of plant growth hormones at the same ratio of cytokinins /auxins conducted to acceptable levels of regeneration.

By the other hand on complex regeneration variants, the rooting process was absent or underwent at low levels.

Using simplified media with reduced levels of macro- and microelements, both rhizogenesis and shoots formation were induced. The *in vitro* response was characterized through the mean number of regenerated shoots/explants and the mean number of neoformed roots/initial inoculum. Despite of low micro- and macroelements levels (1/2 and ¼ of MS salts), the modified MS basal medium composition proved to be beneficial for *D. nardiformis*, plants could be efficiently regenerated and rooted and regenerative aggregates can also be propagated as short-term cultures. The need of the supplementary transfer on particular rooting medium is also eliminated.

Using simplified culture media, this species can be efficiently micropropagated and maintained as active tissue cultures, able to supply viable plants whenever it is necessary.

Keywords: *Dianthus nardiformis*, *in vitro* optimization, complex and simplified culture media.

INTRODUCTION

The plant genetic resources underwent a dramatically decline owing to natural factors such as genetic erosion, abiotic factors (climatic changes, deserts expansion, different cataclysms) and to biotic factors (pests and diseases). Also important impacts have the anthropic factors (deforestations and cultivated lands expansion, the overexploitation of the natural products, the industry and infrastructures development, the pollution).

Between 1996 and 2004, 8321 species were new introduced in the Red List of IUCN, being registered an increasing with 60% of the number of the endangered ones, for this reason complex and complementary measures being necessary to be adopted [19]. In Romania, 14.5 % from the Romanian Flora are considered endangered taxa [7].

Besides the classical *ex situ* conservation approaches, alternative strategies for *ex situ* conservation including the *in vitro* cultures can significantly sustain and complement the *in situ* conservation. The plant biotechnology has been strongly involved in *ex situ* conservation of plant genetic resources for several years being recognized the important role [1, 2, 4, 8, 9, 11, 13, 19, 21, 22]. The *in vitro* plant manipulation allows the production of valuable, healthy plant material with high conservative value such as meristems, apexes and somatic embryos in relatively short time [4].

Using the biotechnological approach, the conservation can be made on short, medium or long term, the biochemical and molecular analyses allowing the characterization of the plant material for *in vitro* collection of plant genetic resources [11]. This kind of preservation needs protocols issued for every particular taxon.

The *ex situ* conservation of different genotypes originated from different locations can ensure the variability of the preserved plant material, the elaboration of reproducible and efficient micropropagation protocols being necessary for the active collection establishment (developed during 1-3 years) [1, 11, 13]. Meanwhile, on *in vitro* protocols depends the production of the material for medium- and long- term preservation and also for different exchanges of plant material and for the repopulations programs.

An important aspect to take into consideration is to avoid as much as possible the use of methods that could induce somaclonal variation, the preserved material has to be genetically identical as donor plants, besides the particular situation where this variation it is desired (low variability in the natural populations).

In Romania, in the last years, many studies concerning *in vitro* cultures for conservative purpose in *Dianthus* endangered species were carried out [3, 5, 6, 12, 14, 16, 20]. The species taken in our study is *Dianthus nardiformis* Janka, a vulnerable, subendemic plant species, growing in Romania and Bulgaria, as a Dobrogean element [7, 18].

Dianthus nardiformis Janka is a perennial, xerophyle species growing on calcareous substrates, with reduced height and red-violet flowers, blossoming in June-august with decorative value.

Our previous researches in this species [15] refer to the *in vitro* culture introduction of the collected plant material from three Romanian Natura 2000 Sites, preliminary tests for *in vitro* reaction had been made. The main problems to be solved were the high

contamination rate of the explants collected from the mature plants and the poor rooting rate.

In the present study, an analysis concerning the *in vitro* behavior using complex – plant growth regulators added media and simplified media with reduced nutrients levels for the optimization of the regeneration and rooting rate was made.

MATERIALS AND METHODS

Plant material previously *in vitro* obtained from 3 individuals growing in the natural habitats from the Măcin National Park, from Allah Bair Hill and Consul Hill [15] was used for testing different culture media.

The initial plant material (shoots fragments and seeds) was sterilized using previously reported protocols [15]. Owing to the fragility of this species, the initial explants consisted in two nodes stem fragments, 5 inocula were cultured on 125 ml jars X 3 repetitions/ variant.

For the optimization of *in vitro* regeneration, taking into account that *D. nardiformis* is a endangered species and we collected a limited plant material, at the beginning, 9 complex variants based on Murashige-Skoog formula [17], supplemented with 30g/l sucrose and B5 vitamins mixture [10], solidified with agar (Duchefa Plant Agar) 8 g/l and pH adjusted at 5.5-5.8 before autoclavation, were tested (Table 1).

Table 1. The composition of complex regeneration media M1-M9 tested in *D. nardiformis.*

Components		Variants								
		M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**M8**	**M9**
Macroelements		MS	MS 1/2	MS 1/2	MS	MS	MS	MS	MS	MS
Microelements		MS	MS 1/2	MS 1/2	MS	MS	MS	MS	MS	MS
B Complex Vitamins		B 5	B5	B5	B5	B5	B5	B5	B5	B5
Growth plant regulators (mg/l)	BAP		0.1	0.1	-	-	-	1	1	-
	Kin	-	-	-	1	0.1	0.1	1	1	-
	Zea	-	-	-	-	-	-	-	-	2
	GA_3	-	-	-	-	-	-	-	0.5	-
	NAA	-	0.01	0.01	0.1	0.01	-	-	0.2	0.2
	2.4- D	-	-		-	-	0.01	0.2	-	-
Other com-pounds (g/l)	Glut	-	-	0.2	-	-	-	-	-	-
	AC	500	-	-	-	-	-	-	-	-
Sucrose (g/l)		30	30	30	30	30	30	30	30	30
Agar (g/l)		8	8	8	8	8	8	8	8	8

Note: MS – Murashige & Skoog medium, B5 – Gamborg vitamins mixture; BAP – 6-benzyl aminopurine; Kin – kinetin; Zea – zeatin; GA_3- gibberellic acid; NAA – α naphthaleneacetic acid; 2.4-D – 2,4-dichlorophenoxyacetic acid; Glut – glutamine; AC – active charcoal.

The simplest composition of M1 variant contained only active charcoal (AC). Taking into account that our previously experience showed that cytokinins and auxins presence added at 1-2 mg/l concentration first and 0.1-0.2 mg/l the second one conducted to good regeneration results through direct shooting in many species including those belonging to *Dianthus* genus, in M2, M3, M5 variants we used very diminished levels (0.1-0.01 mg/l) of cytokinins (N^6- amynopurine (BAP), kinetin (Kin) and zeatin (Zea), and auxins (α naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid (2,4-D) in 10/1 ratio cytokinis/auxins. M4, M7, M8, M9 variants were added with complex growth plant regulators combinations at usual concentrations beneficial for *Dianthus* micropropagation (1-2 mg/l for cytokinins and 0.1-0.2 for auxins). M8 medium was also supplemented with gibberellic acid (GA_3) at 0.5 mg/l. The simplest medium variant devoid of plant regulators (M1) was taken as control. The results of regeneration were also compared with the M7 variant with the best registered *in vitro* response.

Taking into account that on complex media was observed the vitrification tendency of shoots and rooting difficulty, for the optimization of *in vitro* cultures concerning the regeneration, for the rooting and for economical reasons, besides of complex media, simplified variants were further tested (R1-R5) (Table 2).

Half and quarter strength MS simplified media and with sucrose level at 20 or 10 g/l and without plant growth regulators excepting R3 variant (with a low level of NAA) were also used. R6 variant with normal MS salt levels and 30 g/l sucrose was taken as control taking into consideration that this variant was used for *D. nardiformis* shoots rooting after the micropropagation step in the first test. All the cultures were maintained in the growth chamber at 2000 lux illumination and 16/8 photoperiod and 25^0C temperature.

Two aspects were evaluated: the regeneration and the rooting process. The *in vitro* behavior was evaluated after 40 days concerning the mean number of new regenerated shoots/inoculum and also the mean number of neoformed roots/explant only in the case of simplified media where the rooting occurred.

Graphic values are expressed as mean values ±SD. Statistical analysis was carried out using PrismDemo software, one way analysis of variance (ANOVA) was used. P value <0.05 were considered as significant. Also significance difference tests (Student-Newman-Keuls and Dunnett) were used.

RESULTS

D. nardiformis had proved a good *in vitro* reactivity [15] - the way of regeneration is the direct morphogenesis, the process having the origin at the lateral meristems of the stem fragments with the

formation of several shoots depending of culture media used. There were some differences concerning the *in vitro* behavior of the *in vitro* cultures on M1-M9 media variants concerning the regeneration, the growth of shoots, the vitrification and rooting (Table 4, Fig.1).

Table 2. The composition of minimal media variants tested for rooting and shooting induction.

Components		Variants					
		R1	R2	R3	R4	R5	R6
Macroelements		MS 1/2	MS 1/2	MS 1/2	MS 1/4	MS 1/4	MS
Microelements		MS 1/2	MS 1/2	MS 1/2	MS 1/4	MS 1/4	MS
B Complex Vitamins		-	-	-	-	-	-
Plant growth regulators (mg/l)	NAA	-	-	0.01	-	-	-
Other compounds (mg/l)	AC	-	500	-	-	-	-
Sucrose (g/l)		20	20	20	20	10	30
Agar (g/l)		8	8	8	8	8	8

Note: MS – Murashige & Skoog medium; NAA – - α naphthalene acetic acid; AC – active charcoal.

The cytokinin dominance has always favoured the development of several buds starting from the lateral meristems of the explants (Fig. 5a,b).

The complex regeneration media (M4, M7, M8, M9) with 10/1 ratio of cytokinins/auxins at normal concentration induced the best regeneration starting from the explants prelevated from mature plants the first 40 days of culture, the mean number of shoots/explant varying between 6.08 and 8.3 (Fig. 4b, 5a, 5b). The result were significant different comparing to M1 variant in the case of M7, M8, M9. The regeneration occured at the similar level in M7, M8, M9, the comparison to the difference of the averages were non significant, but at lower level on M4 variant (Table 3). In the case of complex variants with higher levels of growth regulators, the vitrification process and the low vigurosity of neoformed shoots was observed. Although, on M8 medium the shoots developed better than those induced on M7 and M9 variants.

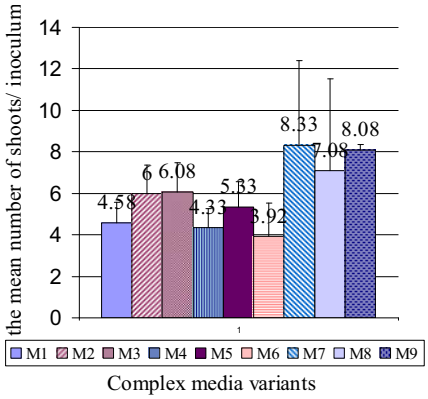

Figure 1. The mean number of shoots / explant regenerated on complex media (M1-M9) (average values with standard deviation).

Table 3. The statistical result concerning the mean number of shoots/ explants on complex media variants.

Statistics evaluation	Media variants								
	M1	M2	M3	M4	M5	M6	M7	M8	M9
$\bar{x}$± Sd	4.58±0.16	6.00±1.96	6.081±1.42	4.33±0.54	5.33±1.11	3.91±1.16	8.33±1.90	7.91±0.50	8.08±2.60
$\bar{x}$± SE	4.58±0.08	6.00±0.98	6.081±0.71	4.33±0.27	5.33±0.57	3.91±0.58	8.33±0.95	7.91±0.25	8.08±1.30
d V-C	-	-1.41 (ns)	-1.50 (ns)	0.25 (ns)	-0.75 (ns)	0.66 (ns)	-3.7 **	-3.33 *	-3.5 *
dV-M7	-3.75 *	-2.33 (ns)	-2.25 (ns)	-4.00 *	-3.00 *	-4.41 **	-	-0.417 (ns)	-0.25 (ns)

Note: Significant at P<0.05; ns- nonsignificant. _
x ± Sd- average± Standard deviation, $\bar{x}$± SE- average± Standard error, dV-C difference between average of variant and control, dV-M7 difference between M7 and other variants.

Table 4. Observations concerning *in vitro* response in *Dianthus nardiformis* on complex regeneration media.

Complex media tested	Observations
M1	short shoots with 2-3 nodes, few developed roots
M2	shoots with 2-4 nodes, without rooting
M3	developed shoots with 2-3 nodes but having tendency of etiolation, without rooting
M4	shoots with 4-5 nodes with tendency of vitrification , without rooting
M5	shoots with 2-3 nodes with 1-2 well developed roots.
M6	Low regeneration rate, high rate of growth of shoots reaching 7 nodes, without root formation
M7	Very good regeneration, but having low growth of shoots at 1-2 nodes, low vitrification, without rooting.
M8	Very good regeneration, shoots at 4-5 nodes, without rooting
M9	Very good regeneration, shorter shoots formation with 2-3 nodes, with vitrification tendency, without roots formation.

In the complex culture media M2, M3, M5, M6 with diminished levels of plant growth regulators, the regeneration process occurred with rates varying between 4 and 6 shoots (Fig.1, Fig. 4a). Although, the variants supplemented with BAP even at low level (M2, M3) prouved to be better than the use of kinetin at the same concentration (M5, M6). Although comparing to M1, the differences of regeneration are not significant. Compared to M7 (the variant of complex medium with the best results), the regeneration was similary on M2 and M3 medium, the differences between the averages being non significant.

In almost complex regeneration media the rhizogenesis process is absent or reduced. In M1 medium, the presence of active charcoal was beneficial for few roots induction. Also on M5 variant supplemented with low level of kinetin (0.1 mg/l) and NAA (0.01 mg/l), 1-2 roots/explant were formed.

Using simplified culture media with reduced salts content (R1-R5), in the second experiment, the root formation underwent at good levels in all variants tested (Fig. 2). Even on R5 variant with low macro- and microelements levels and decreased sucrose level at 10 g/l, the rooting occurred at rates closed to the Control (R6). The presence of low concentration of auxin (NAA at 0.01 mg/l) in R3 variant favored good rooting.

The differences concerning the rooting process of the simplified medium variants compared to R6 medium taken as control were not significant (Table 5).

Concerning the capacity to support the regeneration process, the simplified variants can sustain the shoots formation at good levels even compared to complex variants of regeneration media (Fig. 6b).

The regeneration occurred at high levels on all simplified variants tested except R5 variant with ¼ MS reduced salts and sucrose at 10g/l, the result were significant different compared to R6 (Table 6).

The best results were registered on R3 and R4 simplified medium variants.

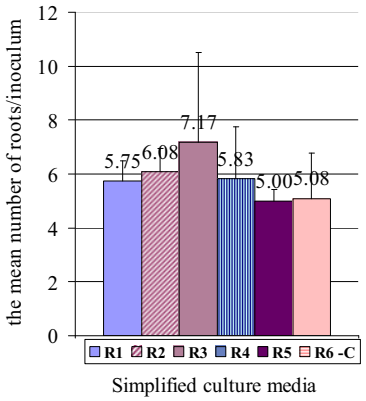

Figure 2. The mean number of roots/ inoculum induced on simplified culture media in *Dianthus nardiformis* (average values with standard deviation).

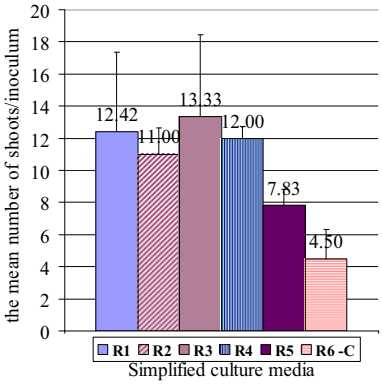

Figure. 3. The number of neoformed shoots on simplified culture media in *Dianthus nardiformis*(average values with standard deviation).

Table 5. Statistical analysis of rooting in *Dianthus nardiformis* on simplified culture media.

Statistic evaluation	Simplified media variants					
	R1	R2	R3	R4	R5	R6
$\bar{x}$± Sd	5.75±0.56	6.08±0.31	5.50±0.57	5.83±0.43	5.0±0.54	5.08±2.16
$\bar{x}$± SE	5.75±0.28	6.08±0.15	5.50±0.28	5.83±0.21	5.0±0.27	5.08±1.083
dV-C	-0.66 (ns)	-1.00 (ns)	0.41 (ns)	0.75 (ns)	0.08 (ns)	-

Note: Significant at P<0.05; ns- nonsignificant.
$\bar{x}$± Sd- average± Standard deviation, $\bar{x}$± SE – average± Standard error, dV-C difference between average of variant and control.

DISCUSSION

In other endangered *Dianthus* species from Romanian Flora have also been reported protocols of regeneration which recommended the use of a cytokinin as BAP or kinetin at 1-2 mg/l level combined with and auxin, usually NAA (0.1 mg/l) [5, 6, 12, 14, 20], but in *D. nardiformis* these variants did not ensure the best regeneration.

For conservative purpose it is important to have an optimized micropropagation protocol to induce a satisfactory regeneration of vigorous and rooted plants and to avoid as much as possible the use of high levels of growth regulators and indirect way of regeneration.

Because the plant material collected from the natural populations was reduced and highly contaminated with bacteria and fungy, we used at the beginning complex regeneration variants to promote increased regeneration rates.

Generally, in *D. nardiformis*, medium MS added with complex combination of plant growth regulators with cytokinin dominance allowed good rates of regeneration (as in case of M7, M8, M9 complex variants), but an inconvenient was the vitrification tendency.

Zeatin presence was stimulatory for regeneration, but being very expensive, the combination of BAP and kinetin could be used with almost similar effect on

regeneration. The presence of 2.4-D at low level in M7 variant had a positive influence on the mean number of shoots/ explant.

By the other hand on these variants, the rooting was absent or underwent at low levels, being necessary the transfer of the shoots on rooting medium.

Using simplified medium variants with macro and micronutrients content of MS basal composition reduced, the regeneration and rooting of the shoots occurred at good levels compared to R6 variant with non reduced salts content and also with complex medium variants.

In *D. nardiformis*, the simplified variants can be suitable for the micropropagation, this species can sustain *in vitro* morphogenesis even on media with reduced salts content.

Table 6. Statistical analysis of neoformed shoots in *Dianthus nardiformis* on simplified culture media.

Statistic evaluation	Simplified media variants					
	R1	R2	R3	R4	R5	R6
$\bar{x}$± Sd	12.4±3.44	11.00±1.96	13.33±1.82	12.00±0.81	7.83±1.59	4.58±0.99
$\bar{x}$± SE	12.4±1.72	11.00±0.98	13.33±0.91	12.00±0.40	7.83±0.79	4.58±0.49
d V-C	-7.83 ***	-6.41 ***	-8.75 ***	-7.41 ***	-3.25 (ns)	-

Note: Significant at P<0.05; ns- nonsignificant.
$\bar{x}$± Sd- average± Standard deviation, $\bar{x}$± SE - average± Standard error, dV-C difference between average of variant and control.

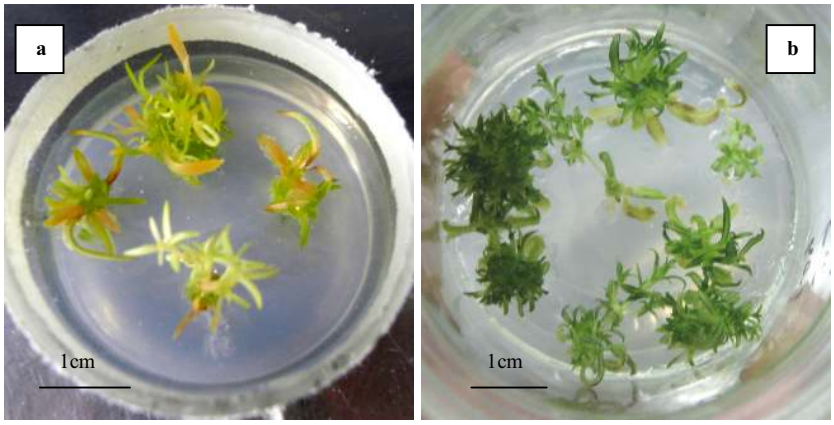

Figure 4. Induction of direct shooting in *D. nardiformis* on M3 variant (**a**); regeneration on M7 medium variant (**b**) after 40 days of culture.

Figure 5. Direct morphogenesis induced on M8 medium variant (**a**) and M9 variant (**b**) after 40 days.

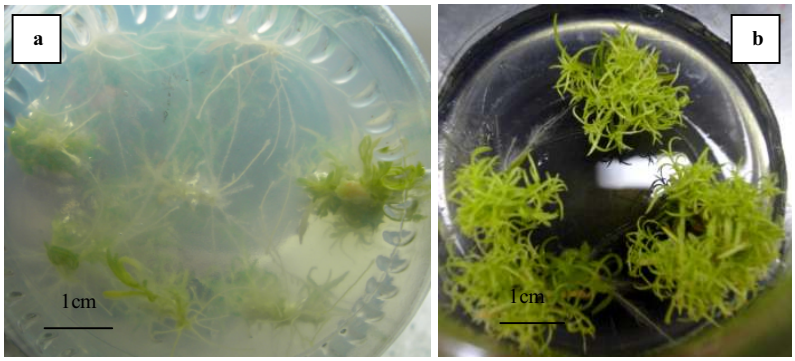

Figure 6. *In vitro* cultures of *D. nardiformis* on simplified media variant after 40 days of culture (**a**) the roots development on R4 medium; (**b**) the regeneration of rooted shoots on R2 medium.

Taking into consideration the economical aspects correlated with the *in vitro* response, we recommended R4 variants with ¼ reduced salts contents, added with 20 g/l sucrose and without any supplementary factor, the difference with R3 variant being non significant.

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