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Original paper

In Vitro **Conservation of Genetic Resources of** *Nymphaea lotus* **var.** *thermalis* **(DC.) Tuzs., an Endangered Plant Species**

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Abstract

Nymphaea lotus var. *thermalis* (DC.) Tuzs. is the main relict hydrobiont that survived the Quaternary glaciations due to warm microclimate ensured by thermal ecosystem of "Peţea stream" Natural Reserve, which presently is the unique habitat hosting this variety. Currently this protected area is in an advanced degree of degradation due to decrease, until the cessation of the main thermal spring that feeds this aquatic ecosystem. The drastic reduction down to 2% in only 10 years critically endangers the survival of the species. The main purpose of this study was to explore ways of using *in vitro* culture techniques for the conservation of thermal water lily, namely the identification of the optimal culture medium, both in terms of plant hormones composition and physical support of type of inoculants. Research has revealed that using liquid culture medium MS62, lacking sustainment support of inoculants, supplemented with 2 mg/L IAA and 2 mg/L Z, resulted in highest germination indices and generated the best rate of caulogenesis, while using Blidar filter paper type bridges stimulated rooting, vitroplantlets' stolon neoformation and development. The danger of imminent extinction of this intraspecific taxon urges further research for species conservation and the need to establish an ex vitro nursery to ensure plant supplies for ecological reconstruction, or to populate other aquatic habitats with similar properties.

Keywords *Nymphaea lotus* var. *Thermalis*, filter paper bridges, *in vitro* conservation, plant biotechnology, "Peţea stream" Natural Reserve.

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Introduction

Water lilies are considered important ornamental plants used for arranging aquatic gardens (Kate and Philman, 1992 – after E.S. SULAIMAN [1]). Furthermore, over the centuries, members of Nymphaeaceae have been used for their pharmaceutical properties. Among these studies we mention those performed on *Nymphaea lotus* well known for its antimicrobial property (O.J. AKINJOGUNLA & al. [2]), and *N. nouchali*, which, also expresses antioxidant and cytotoxic effects (A.A. SIKDER & al. [3]). Moreover, *N. candida* has proven analgesic, antioxidant, liver protection and immune-active properties (J. ZHAO & al. [4]), while *N. alba* expresses antidiarrheic (A. BOSE & al. [5]) and antidepressant roles (A. BOSE & al. [6]).

N. lotus var. *thermalis* (DC) Tuzs. (syn. *Nymphaea thermalis* DC.) is considered an important glacial relict hydrobiont, it belongs to the thermal Nature Reserve "Peţea stream", located in the locality 1 Mai, Bihor county, Romania (Figure 1). In the warm waters of the Peţea stream is the only place in the world where this variety spontaneously survives (V.M. DANCIU [7]).

"Peţea stream" Reserve is registered as a protected area since 1932 (V.M. DANCIU [8]). Based on the Law 5/2000 it is accepted as a IVth protected area category according to standards of International Union for Nature Conservation (IUCN). Since 2007 the reserve is also acknowledged as a protected area of community interest, part of the European ecological network Natura 2000 in Romania (Natura 2000 site ROSCI0098). Among the endemic species, unique for habitats found in the Peţea stream, besides the thermal water lily, the *Melanopsis parreyssi* relict snail and a subspecies of rudd, *Scardinius racovitzai* are also relevant to be mentioned. The reserve is considered an oasis of subtropical vegetation in a temperate zone (T. TOFAN [9]).

The thermal water lily belongs to the Nymphaeales order, respectively the Nymphaeaceae family, which includes about 50-60 species distributed in the tropics towards the temperate zone (Halijah, 2000 – quoted E.S. SULAIMAN [1]). The Nymphaeaceae species are considered as being the ancestral flowering plants, related to the *Cycadales* order (Master, 1974 – quoted E.S. SULAIMAN [1]). Although, it is known since 1798 (i.e. it was described by Conrad as a variety), only in 1908 a more precise classification of the thermal lily based on the Nile lotus was made, the classification that gives its current name (J. TUZSON [10]). Today, there are in-depth studies that provide evidences to support the idea that the *thermalis* variety derives from *lotus* species of the *Nymphaea* genus (T. BORSCH & al. [11]; P. POCZAI & al., [12]).

Regarding the optimum abiotic environment that is necessary for the survival, growth and development of the thermal water lily plants, certain habitat conditions must be met. Following the species observation in the wild, it was determined that for its optimal development, it needs water with a temperature between +20°C and +34°C, a water depth between 45-50 cm, exposure to the sun, a rich mud substrate, low concentration of $CaCO_3$ and an alkaline pH (C. OLTEANU-COSMA [13]).

Over time, there have been a number of *in situ* studies on thermal water lily, of which we mention that of Tuzson made in 1908 (J. TUZSON [10]). Also, studies carried out over several years by Opris were noted, who in 2000 issued the hypothesis that the thermal lily is a species which survived the last ice ages, a theory supported by the seeds discovered in fossil flora at Granocz (Czech Republic) (T. OPRIS [14]).

During the past decade several conservation strategies were developed mainly in the terms of *in situ* and *ex situ* conservation. Among the *ex situ* strategies, biotechnologies including *in vitro* techniques provide an important tool for endangered plant conservation (L. FODORPATAKI & al. [15]; Z.O. KISS & al. [16]; A. PĂUNESCU [17]). In the last two decades, several scientific achievements have been published on biotechnology application involving different varieties of water lilies. Thus, in 1990, a study was conducted to analyse the *in vitro* behavior of 3 mm explants obtained from mature leaves of *Nymphaea* 'Dauben' species. One result of the study emphasized that thidiazuron (TDZ) enhances the development of shoots epifili (M. JENKS & al. [18]). Later , the same team established a protocol for making a rapid caulogenesis in aquatic plants (*Nymphoides indica*), using explants' stems grown on basic MS62 medium supplemented with different concentrations of growth hormones, maximum regeneration of shoots having an 80% frequency (M.A. JENKS & al., 2000).

In 1990 Swindells tried to establish a culture of *Nymphaea* spp. using buds originating from rhizomes, leaf explants and stem fragments, but the experiment failed due to extremely high rates of infections (P.R. SWINDELLS [20]).

Later, in 1994, for shoot proliferation originating from explants consisting of fragments of rhizomes from *Nymphaea* 'James Brydon' hybrid, MS62 culture medium supplemented with growth regulators was tested. The obtained seedlings developed root systems of various sizes after they were transferred to culture media containing charcoal (P. LAKSHMANAN [21]).

Subsequent researches targeted the *in vitro* multiplication in the *Nymphaea* genus, but only for *N. alba* L. they used seeds. The best results were obtained on the classical culture medium MS62, supplemented with hormones 6-benzylaminopurine (BAP) and indole-3-acetic acid (IAA). It was also emphasized the role of TDZ in triggering seed germination in *N. alba*, germination rate being of 51.37% (S. SUMLU [22]).

The survival, growth and development of any plant species is achieved in close correlation with the habitat in which it lives. Presently the "Peţea stream" Natural Reserve, is in an advanced state of degradation (Fig. 1C).

The causes of the decline of this unique habitat in Europe are of anthropic origin and are present ever since human settlements and the development of a civilization appeared in this area (A. MAROSSY [23]). However, since two decades ago, the Peţea lake undergoes intense

eutrophication and is in an intermediate stage of clogging (V. ŞOLDEA [24]). Moreover, in the last decade the level of water decreased and its temperature changed drastically, as a result of over-exploitation disrupting the natural resilience of the water resource from the geothermal acquifer that feeds the sublacustrine springs of the reserve (G. PAAL [25]; I. COHUT [26]). In the winter of 2012 the Petea lake froze for the first time in the last 200 years, and this situation continued every year since then.

In the present context of the fast deterioration of the natural habitat, intense conservation and monitoring programs should be in place for Peţea Lake, with emphasis on all endemic species. Given the decreasing number of thermal lotus individuals, one of the very first tasks was to look for measures of saving from extinction this endemic species, such as plant *in vitro* micropropagation.

The aim of this study was to initiate *in vitro* cultures for *N. lotus* var. *thermalis*, to create a stock of plants, that can be reintroduced in the wild, with the purpose of restoring the population of thermal lilies from the "Petea" Natural Reserve and / or populating other natural or similar

Material and methods

The plant material and the conditions of inoculation and growth

The used plant material consisted in thermal waterlily seeds, taken from fruits harvested from "Big Eye" Lake of "Peţea Stream" Natural Reserve within the Natura 2000 site ROSCI0098, "Peţea Lake", in late September and early October 2013. After harvesting, the fruits were placed in three pots of 10 liter volume containing 3-5 cm substrate of sludge filled wuth thermal water, and maintained at a constant temperature of 26-28°C.

The used seeds were selected to be not damaged and uniform in size, shape and color. They were kept under tap water for 20 min and were subsequently subjected to disinfection with sodium hypochlorite 5%, as a commercial product *Ace automat* (Procter & Gamble) for 6 min. The soaked seeds were rinsed with 6 series of sterile distilled water for 2 min. / series, shaking them gently to remove any sterilizing agent.

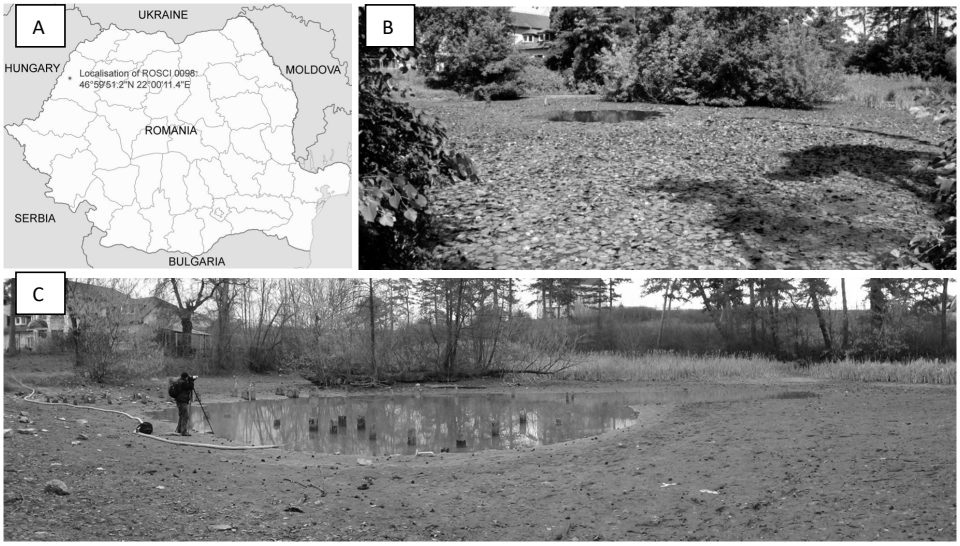

Figure 1. Location (A) and overall look of the "Big Eye" Lake belonging to "Peţea Stream" Reserve (within the Natura 2000 site ROSCI0098 "Peţea Lake"), prior to human impact to which it has been aggressively submitted in the last 5 years – in June 2011 (B) (Photo: R. Huza) and after it occurred – in December 2012 (C) (Photo: C.F. Blidar).

artificial thermal habitats with this relict plant species. The main challenge was to identify the optimal culture medium for the initiation of *in vitro* cultures, both in terms of plant hormones and type of physical sustainment support for the *in vitro* plantlets. Therefore, the present research supports the conservation and sustainable use of the thermal lily in its own natural habitat by using biotechnology as a tool.

The basal Murashige-Skoog (MS62) (T. MURASHIGE & al. [27]) culture medium was used in this experiment with two types of physical support substrates of the inocula: liquid (V_L), and the second variant using Blidar filter paper bridges (V_{PB}) (C.F. BLIDAR [28]). Three experimental types of culture media have been used regarding the content of plant hormones: V_0: MS62 free of growth regulators;

V_1: MS62 supplemented with 1 mg/L IAA (5.70 μM) + 1 mg/L zeatine (Z) (4.56 μM); V_2: MS62 supplemented with 2 mg/L IAA (11.41 μM) + 2 mg/L Z (9.12 μM). As a result, six experimental culture media were set, each consisting of 100 *in vitro* plants.

After inoculation, the pots were placed in the vegetation room, the growth conditions were identical for all experimental variants. The average temperature was of 24-26°C, the lighting was supplied by white fluorescent day-light tubes (6500K), with an intensity of 60 μM $m^{-2}s^{-1}$ PAR, and a photoperiod of 16 h light / 24 h.

Morphometry

The germination and growth of *in vitro* plants were analysed using a series of biometric and gravimetric parameters: the germination rate (the number of seeds from a lot able to germinate may be expressed as the percentage of the seeds that germinate in the lot), number of roots, length of the roots, number of stolons, length of the stolons, number of leaflets, length of the stem, length of the leaf blade, fresh weight (mg) and dry weight (mg). Observations were made at each 20 days, until the 60th day of the *in vitro* cultures as of the time of the experiment implementation.

Statistical analysis: V_0L was considered the control group and the registered values were taken as references for other experimental variants. All statistical analyses were made using Microsoft Excel; values are significantly different at $P<0.05$ according to the Student's *t*-test. The experiment was repeated three times.

Results and discussions

The tested culture media, with or without Blidar type filter paper bridges, proved to be effective in the growth and development of *N. lotus* var. *thermalis* seedlings. It has been found that the use of the liquid culture media without filter paper bridges favoured the sprouting of a larger number of inocula and also initiated caulogenesis. Instead, by using the liquid media provided with filter paper bridges, the most intense rooting was induced, and the formation of stolons on plantlets was also favoured.

Morphological and biometric aspects observed at 20 days

After 20 days from the initiation of the vitrocultures, considering the general and morphological aspects, all inocula presented 2-4 leaflets, their color being more intense for the *in vitro* plants cultivated on liquid experimental variants, lacking filter paper support ("L" Series), compared to those growth on environmental variants that were provided with the sustainment support of the inoculants ("PB" Series). In terms of the size of the vitroplantlets, the largest and most vigorous seedlings were obtained on the liquid culture medium supplemented with 2 mg/L IAA + 2 mg/L Z, the presence of these plant hormones stimulating both rooting (i.e. incipient at this time of the observations) and caulogenesis. In comparison, the vitroplantlets obtained in the experimental variants of the "L" series presented significantly higher sizes, but the "PB" series registered a higher morphological and physiological differentiation, the seedlings of the experimental variant V_2PB initiated the formation of absorbing root hairs. In the V_2L experimental variant, a more intense red-violet pigmentation was observed, which suggested the presence of anthocyanins in the cells.

The first biometric aspect that differentiated the thermal water lily *in vitro* plants grown in the two experimental series, was given by the germination faculty. In comparison to the control (V_0L), there was an increased sprouting of the seeds placed in the liquid culture medium supplemented with 2 mg/L IAA + 2 mg/L Z (V_2L), the percentage difference compared to the control being 30.5% (Table 1A). With respect to the number of new roots, identical maximum values were registered in both types of media – with and without sustainment support given by the filter paper bridge – but containing 1 mg/L IAA + 1 mg/L Z, respectively V_1PB and V_1L experimental variants, the differences being statistically significant (Table 1B). Regarding the root length, the highest values were registered in case of V_1L variant (1 mg/L IAA + 1 mg/L Z), compared with the liquid culture medium, without growth regulators, namely the control (V_0L). The increase was over three times, the data being backed up statistically (Table 1C). At 20 days, there have been not recorded stolons. The number of leaflets and the petiole length reached their highest values on the V_1L variant compared to control (V_0L), the percentage differences were of 120% and 74.1% (Table 1D and 2A). Within the same "L" series, there have been recorded the highest values for the leaf blade length, namely in the presence of 2 mg/L IAA and 2 mg/L Z (var. V_2L), followed by a small difference (i.e. only 0.2 mm in absolute values) by the data marked on the V_1L variant (Table 2B). With respect to the gravimetric parameters of fresh and dry weights, higher values of the inoculi placed in liquid medium ("L" Series) have been recorded, under conditions of hypoxia, compared to those placed on the filter paper bridge ("PB" series). The highest accumulation of plant biomass was registered in the presence of the culture medium containing 2 mg/L of IAA and Z (V_2L), the increases of accumulation compared to the control (V_0L) being of 250% for fresh weight, respectively 60% on a dry weight basis (Table 3A and 3B).

Morphological and biometric aspects observed at 40 days

Morphologically, at 40 days of *in vitro* cultivation, the seedlings grown on both experimental series ("L" and "PB") presented an intense caulogenesis and developed a large number of leaflets. In terms of the size of the leaflets, the highest values were recorded in case of explants cultivated on liquid experimental variants, without filter paper support ("L" series), these having the form of a spear and a more intense green color. At the same time, the seedlings of the "PB" series developed a hastate leaf as a sign of advanced development based on "L" series, their color being lighter green. As of this date of the observations, the appearance of green-albino stolons was noted, with lengths up to 5 mm

for liquid experimental variants ("L" series), or up to 10 mm in the experimental variants provided with filter paper bridges (belonging to the "PB" series).

Regarding the number of roots and their length, similarities between the inocula placed in the two types of media were recorded: liquid and liquid provided with filter paper bridges. The longest roots were recorded in V_1L and V_0PB variants, the increase compared to the control (V_0L) was of only 3.7%. The number of stolons and their length registered values higher by 50% and respectively by 43.2% for *in vitro* plants generated on filter paper bridges without growth hormones (V_0PB), compared to V_0L (control version) (Table 2C and 2D). The highest values of the length of the stem and the length of the leaf blade were determined for the vitroplants submerged in liquid medium supplemented with 2 mg/L IAA + 2 mg/L Z (V_2L), the differences to the control lot being significant statistically (Table 1D, 2A and 2B). Following the above mentioned results, it was expected that the highest accumulation of plant biomass, both fresh and dry weights, to be registered in the same experimental variant (V_2L), the percentage differences from the control being significant (178% for fresh weight and 200% for dry weight) (Table 3A and 3B).

Morphological and biometric aspects observed at 60 days

After 60 days, the vitroplantlets of *N. lotus* var. *thermalis* were characterized by strong vitality, marked by a broad caulogenesis, developing a large number of leaflets, whose dimensions were higher compared to those recorded during the observations at 40 days, both in the test series without filter paper bridges ("L"), and that with sustainment support of the inocula ("PB" series) (Fig. 2A-D). The most intense organogenesis was recorded in V_2L variant (liquid medium free of filter paper support and supplemented with 2 mg/L IAA + 2 mg/L Z), both regarding the rooting and the caulogenesis. The red-violet pigmentation due to the presence of anthocyanins was mostly maintained in the younger leaves and in the petioles of the mature leaves, especially for the seedlings obtained in variants with filter paper support (Fig. 2B). At this time of the observations, the differences in terms of the vitroplantlet leaflet form in the case of the two experimental series ("L" and "PB"), were obvious in the liquid medium without sustainment support (i.e. in which the vitroplantlets were submerged), spear shape was recorded (rarely linear),

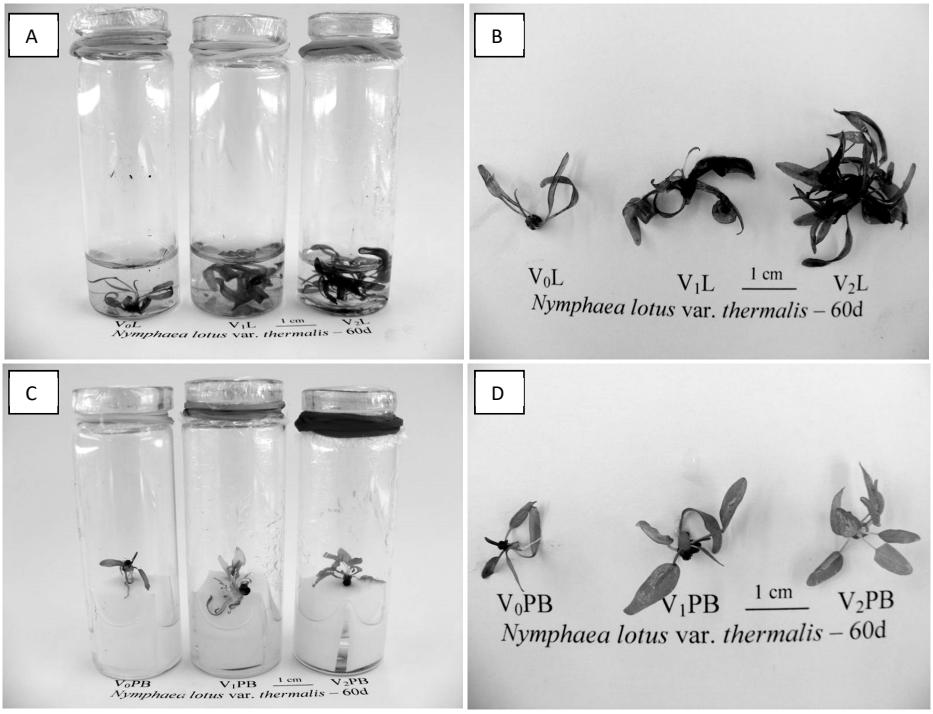

Figure 2. General comparative aspects of *in vitro* plants of the *N. lotus* var. *thermalis* grown in the MS62 culture medium, liquid (**L**) (A, B) and the liquid culture medium provided with filter paper supportl (**PB**) (C, D), at 60 days after the implementation of the experiments, where: $\mathbf{V_0}$ - control variant, without phytohormones; $\mathbf{V_1}$ - MS62 + 1 mg/L IAA + 1 mg/L Z; $\mathbf{V_2}$ - MS62 + 2 mg/L IAA + 2 mg/L Z.

Table 1. Statistical processing of the *germination faculty* (A), *roots number* (B), *root length* (C), *leaf number* (D), measured in the *in vitro* seedlings of *N. lotus* var. *thermalis* in "**L**" series – liquid MS62 culture medium and "**PB**" series – liquid MS62 culture medium with filter paper bridges, where **V_0**: MS62 without phytohormones; **V_1**: MS62 + 1 mg/L IAA + 1 mg/L Z; **V_2**: MS62 + 2 mg/L IAA + 2 mg/L Z.

	No. of days	Control lot	Statistical data		Variants	Statistical data				Significance
			X ± Sx	s^2		X ± Sx	s^2	±d	%	
A. Germination faculty (%)	20	V_0L	38.3 ± n/a	n/a	V_1L	39.7 ± n/a	n/a	1.4	3.6	n/a
					V_2L	50 ± n/a	n/a	11.7	30.5	n/a
					V_0PB	17.9 ± n/a	n/a	-20.4	-53.2	n/a
					V_1PB	45 ± n/a	n/a	6.7	17.4	n/a
					V_2PB	45 ± n/a	n/a	6.7	17.4	n/a
B. Roots number	20	V_0L	0.3 ± 0.47	0.23	V_1L	0.8 ± 0.40	0.16	0.5	166.7	***
					V_2L	0.5 ± 0.58	0.34	0.2	66.7	ns
					V_0PB	0.5 ± 0.64	0.41	0.2	66.7	ns
					V_1PB	0.8 ± 0.65	0.43	0.5	166.7	***
					V_2PB	0.3 ± 0.62	0.38	0	0	ns
	40	V_0L	1 ± 0.33	0.11	V_1L	0.8 ± 0.40	0.16	-0.2	-20	**
					V_2L	0.8 ± 0.48	0.23	-0.2	-20	*
					V_0PB	0.7 ± 0.77	0.60	-0.3	-30	*
					V_1PB	1 ± 0.47	0.22	0	0	ns
					V_2PB	0.8 ± 0.69	0.48	-0.2	-20	ns
	60	V_0L	1.1 ± 0.42	0.17	V_1L	1.1 ± 0.32	0.10	0	0	ns
					V_2L	1.8 ± 0.76	0.57	0.7	63.6	***
					V_0PB	1 ± 0.63	0.39	-0.1	-9.0	ns
					V_1PB	1.3 ± 0.55	0.31	-0.2	-18.1	*
					V_2PB	1.2 ± 0.81	0.66	0.1	9.0	ns
C. Roots length (mm)	20	V_0L	0.7 ± 0.46	0.21	V_1L	2.5 ± 1.3	1.69	1.8	257.1	***
					V_2L	0.7 ± 0.80	0.64	0	0	ns
					V_0PB	1 ± 1.26	1.59	0.3	42.8	ns
					V_1PB	1.2 ± 0.91	0.83	0.5	71.4	***
					V_2PB	1.1 ± 1.85	3.45	0.4	57.14	ns
	40	V_0L	2.7 ± 0.65	0.42	V_1L	2.8 ± 1.46	2.16	0.1	3.7	ns
					V_2L	0.8 ± 0.70	0.49	-1.9	-70.4	***
					V_0PB	2.8 ± 2.68	7.19	0.1	3.7	ns
					V_1PB	1.8 ± 1.09	1.18	-0.9	-33.3	***
					V_2PB	1.3 ± 1.80	3.25	-1.4	-51.9	***
	60	V_0L	3 ± 0.89	0.80	V_1L	2.9 ± 1.28	1.64	-0.1	-3.3	ns
					V_2L	1 ± 0,56	0.32	-2	-66.7	***
					V_0PB	3.2 ± 2.28	5.24	-0.2	6.7	ns
					V_1PB	3.4 ± 0.83	0.70	0.4	13.3	*
					V_2PB	1.7 ± 1.8	3.24	-1.3	-43.3	***
D. Leafs number	20	V_0L	1.5 ± 0.89	0.79	V_1L	3.3 ± 0.67	0.46	1.8	120	***
					V_2L	2.4 ± 1.41	1.99	0.9	60	**
					V_0PB	1 ± 0.56	0.31	-0.5	-33.3	**
					V_1PB	1.5 ± 1.02	1.04	0	0	ns
					V_2PB	1.8 ± 0.73	0.54	0.3	20	*
	40	V_0L	4 ± 0.72	0.53	V_1L	7.3 ± 0.79	0.63	3.3	82.5	***
					V_2L	7.8 ± 0.7	0.49	3.8	95	***
					V_0PB	4.5 ± 1.02	1.06	0.5	12.5	*
					V_1PB	6.5 ± 1.32	1.75	2.5	62.5	***
					V_2PB	7 ± 0.83	0.70	3	75	***
	60	V_0L	5.7 ± 0.60	0.37	V_1L	8 ± 0.82	0.67	2.3	40.4	***
					V_2L	15.7 ± 1.73	2.99	10	175	***
					V_0PB	5.7 ± 1.07	1.16	0	0	ns
					V_1PB	7 ± 1.38	1.92	1.3	22.8	***
					V_2PB	9 ± 0.96	0.92	3.3	57.9	***

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – non applicable.

Table 2. Statistical processing of the *petiole length* (A), *blade length* (B), *stolons number* (C), *stolons length* (D), measured in the *in vitro* seedlings of *N. lotus* var. *thermalis* in "**L**" series – liquid MS62 culture medium and "**PB**" series – liquid MS62 culture medium with filter paper bridges, where **V_0**: MS62 without phytohormones; **V_1**: MS62 + 1 mg/L IAA + 1 mg/L Z; **V_2**: MS62 + 2 mg/L IAA + 2 mg/L Z.

	No. of days	Control lot	Statistical data		Variants	Statistical data				Significance
			X ± Sx	s^2		X ± Sx	s^2	±d	%	
A. Petiole length (mm)	20	V_0L	2.7 ± 1.46	2.13	V_1L	4.7 ± 1.21	1.48	2	74.1	***
					V_2L	4.6 ± 2.18	4.76	1.9	70.4	***
					V_0PB	1 ± 0.44	1.13	-1.7	-63	***
					V_1PB	2.2 ± 1.09	1.18	-0.5	-18.5	ns
					V_2PB	1.8 ± 0.68	0.46	-0.9	-33.3	**
	40	V_0L	4.9 ± 0.82	0.68	V_1L	5.7 ± 0.82	0.68	0.8	16.3	***
					V_2L	6.8 ± 1.78	3.18	1.9	38.8	***
					V_0PB	3.5 ± 0.94	0.89	-1.4	-28.6	***
					V_1PB	3.7 ± 0.98	0.97	-1.2	-24.5	***
					V_2PB	3.9 ± 0.92	0.97	-1	-20.4	***
	60	V_0L	5.6 ± 1.07	1.16	V_1L	7.4 ± 0.90	0.81	1.8	32.1	***
					V_2L	7 ± 1.31	1.73	1.4	25	***
					V_0PB	3.7 ± 0.86	0.74	-1.9	-33.9	***
					V_1PB	4.1 ± 1.20	1.45	-1.5	-26.8	***
					V_2PB	4.2 ± 0.87	0.76	-1.4	-25	***

	No. of days	Control lot	Statistical data		Variants	Statistical data				Significance
			X ± Sx	s^2		X ± Sx	s^2	±d	%	
B. Blade length (mm)	20	V_0L	3.3 ± 1.66	2.76	V_1L	8.5 ± 1.67	2.81	5.2	157.6	***
					V_2L	8.7 ± 4.24	18.03	5.4	163.6	***
					V_0PB	1 ± 0.44	0.19	-2.3	69.7	***
					V_1PB	3.8 ± 1.89	3.57	0.5	15.2	ns
					V_2PB	3.3 ± 1.70	2.90	0	0	ns
	40	V_0L	8.1 ± 1.73	3.02	V_1L	13.7 ± 1.63	2.68	5.6	69.1	***
					V_2L	15.8 ± 2.77	7.71	7.7	95.1	***
					V_0PB	5.9 ± 1.41	1.99	-2.2	-27.2	***
					V_1PB	4.7 ± 1.22	1.49	-3.4	-42	***
					V_2PB	6.9 ± 1.77	0.92	-1.2	-14.8	**
	60	V_0L	9.1 ± 1,55	2.41	V_1L	14.4 ± 1.62	2.65	5.3	58.2	***
					V_2L	17 ± 2.52	6.39	7.9	86.8	***
					V_0PB	6.7 ± 1.45	2.12	-2.4	-26.4	***
					V_1PB	6 ± 1.41	1.99	-3.1	-34.1	***
					V_2PB	7.2 ± 2.7	7.32	-1.9	-20.9	***

	No. of days	Control lot	Statistical data		Variants	Statistical data				Significance
			X ± Sx	s^2		X ± Sx	s^2	±d	%	
C. Stolons nummber	40	V_0L	1.0 ± 0.51	0.26	V_1L	0.9 ± 0.44	0.19	-0.1	-10	ns
					V_2L	0.8 ± 0.48	0.15	-0.2	-20	ns
					V_0PB	1.5 ± 0.76	0.57	0.5	50	**
					V_1PB	0.7 ± 0.55	0.31	-0.3	-30	*
					V_2PB	0.5 ± 0.57	0.32	-0.5	-50	***
	60	V_0L	1.7 ± 0.95	0.90	V_1L	1.3 ± 0.73	0.54	-0.4	-23,5	*
					V_2L	1.2 ± 0.77	0.60	-0.5	-29.4	**
					V_0PB	2.3 ± 0.68	0.47	0.6	35.3	***
					V_1PB	1 ± 0,49	0.24	-0.7	-41.8	***
					V_2PB	0.8 ± 0.62	0.39	-0.9	-52.9	***

	No. of days	Control lot	Statistical data		Variants	Statistical data				Significance
			X ± Sx	s^2		X ± Sx	s^2	±d	%	
D. Stolons length (mm)	40	V_0L	3.7 ± 1.85	3.44	V_1L	3.5 ± 1.77	3.13	-0.2	-5.3	ns
					V_2L	2.3 ± 1.43	2.05	-1.4	-37.8	***
					V_0PB	5.3 ± 1.62	2.63	1.6	43.2	***
					V_1PB	3.3 ± 2.18	4.79	-0.4	-10.8	ns
					V_2PB	1.8 ± 1.86	3.48	-1.9	-51.4	***
	60	V_0L	7.3 ± 1.44	2.07	V_1L	5.6 ± 1.74	3.04	-1.7	-23.3	***
					V_2L	5.2 ± 2.19	4.80	-2.1	-28.8	***
					V_0PB	6.3 ± 1.35	1.84	-1	-13.7	**
					V_1PB	4.4 ± 1.40	1.96	-2.9	-39.7	***
					V_2PB	2.3 ± 1.46	2.15	-5	-68.5	***

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – non applicable.

Table 3. Statistical processing of the *fresh weight* (A) and *dry weight* (B) measured in the *in vitro* seedlings of *N. lotus* var. *thermalis* in "**L**" series – liquid MS62 culture medium and "**PB**" series – liquid MS62 culture medium with filter paper bridges, where $\mathbf{V_0}$: MS62 without phytohormones; $\mathbf{V_1}$: MS62 + 1 mg/L IAA + 1 mg/L Z; $\mathbf{V_2}$: MS62 + 2 mg/L IAA + 2 mg/L Z.

	No. of days	Control lot	Statistical data		Variants	Statistical data				Significance
			X ± Sx	s^2		X ± Sx	s^2	±d	%	
A. Fresh weight (mg)	20	V_0L	5.0 ± n/a	n/a	V_1L	14.9 ± n/a	n/a	9.9	198	n/a
					V_2L	17.5 ± n/a	n/a	12.5	250	n/a
					V_0PB	1.7 ± n/a	n/a	-3.3	-66	n/a
					V_1PB	4.4 ± n/a	n/a	-0.6	-12	n/a
					V_2PB	4.5 ± n/a	n/a	-0.5	-10	n/a
	40	V_0L	21.4 ± n/a	n/a	V_1L	43.6 ± n/a	n/a	22.2	103.7	n/a
					V_2L	59.5 ± n/a	n/a	38.1	178	n/a
					V_0PB	8.5 ± n/a	n/a	-12.9	-60.3	n/a
					V_1PB	12.9 ± n/a	n/a	-8.5	-39.7	n/a
					V_2PB	17.7 ± n/a	n/a	-3.7	-17.3	n/a
	60	V_0L	37.3 ± n/a	n/a	V_1L	69.1 ± n/a	n/a	31.8	85.3	n/a
					V_2L	80.1 ± n/a	n/a	42.8	114.7	n/a
					V_0PB	11.3 ± n/a	n/a	-26	-69.7	n/a
					V_1PB	26.9 ± n/a	n/a	-10.4	-27.9	n/a
					V_2PB	21.9 ± n/a	n/a	-15.4	-41.3	n/a

	No. of days	Control lot	Statistical data		Variants	Statistical data				Significance
			X ± Sx	s^2		X ± Sx	s^2	±d	%	
B. Dry weight (mg)	20	V_0L	0.5 ± n/a	n/a	V_1L	0.6 ± n/a	n/a	0.1	10	n/a
					V_2L	0.8 ± n/a	n/a	0.3	60	n/a
					V_0PB	0.5 ± n/a	n/a	0	0	n/a
					V_1PB	0.5 ± n/a	n/a	0	0	n/a
					V_2PB	0.7 ± n/a	n/a	0.2	40	n/a
	40	V_0L	3.1 ± n/a	n/a	V_1L	7.8 ± n/a	n/a	4.7	151.6	n/a
					V_2L	9.3 ± n/a	n/a	6.2	200	n/a
					V_0PB	1.2 ± n/a	n/a	-1.9	-61.3	n/a
					V_1PB	2.3 ± n/a	n/a	-0.8	-25.8	n/a
					V_2PB	2.1 ± n/a	n/a	-1	-33.3	n/a
	60	V_0L	3.5 ± n/a	n/a	V_1L	8.2 ± n/a	n/a	4.7	134.3	n/a
					V_2L	9.4 ± n/a	n/a	5.9	168.6	n/a
					V_0PB	2.2 ± n/a	n/a	-1.3	-37.1	n/a
					V_1PB	3.1 ± n/a	n/a	-0.4	-11.4	n/a
					V_2PB	3.3 ± n/a	n/a	-0.2	-5.7	n/a

Note: X ± Sx [average (cm) ± standard deviation]; s^2 – variance; ±d – difference to the control lot in absolute values; % – difference to the control lot in percentage values; based on *p* values (significance of difference to control lot): ns – no significant difference ($p>0.1$), * - low significant difference ($0.05<p\leq0.1$), ** - significant difference ($0.01<p\leq0.05$), *** - very significant difference ($p\leq0.01$); n/a – non applicable.

while in the variants where the seedlings grew and developed on filter paper support, the shape of the leaves was hastate and sometimes even sagittate.

Regarding the number of roots, the highest value was recorded in the variant V_2L, due to the presence in the culture medium of 2 mg/L IAA + 2 mg/L Z (Table 1B). The presence of filter paper bridge has facilitated the increase of the root length, the highest values being recorded in V_1PB variant with 13.3% increase compared to control. The support of the inocula at the surface of the liquid medium through filter paper bridges lead to the stimulation of stolon differentiation and development, the increase was by 35.3% higher than in the control (V_0L), with no addition of plant hormones (var. V_0PB) (Table 2C). The lack of hormones also caused the recording of the highest values of the stolons length, but in this case, the maximum values were scored in conditions of hypoxia (var. V_0PB) (Table 2D). The number of leaflets, the petiole length, as well as the leaf blade length had higher values for the vitroplantlets cultivated in liquid medium, compared to those on the filter paper bridge, their appearance and color varying depending on the amount of used hormones. Thus, in case of the V_2L the highest values of leaf number and leaf blade length were recorded (i.e. 175% and 86.8% compared to control – Table 1D and 2B). Regarding fresh weight and dry weight, the trend noticed during the observations at the 20th day and the 40th day was kept, both values reaching the highest values into *in vitro* plants arranged in liquid medium supplemented with 2 mg/L IAA + 2 mg/L Z (V_2L), the percentage differences compared to the control group (V_0L) being of 114.7% for fresh weight, respectively of 168.6% for the dry weight (Table 3A and 3B).

The *in vitro* development of the species belonging to the *Nymphaea* genus has been relatively poorly studied, but for the *N. lotus* var. *thermalis* relict variety there are no studies regarding the *in vitro* behavior and multiplication through plant biotechnology techniques. Since the state of degradation that exists in "Peţea stream" Natural Reserve, the only habitat where the thermal lily spontaneously vegetates, questions the future survival of this extremely

important endemism for the world's genetic resources, its conservation in laboratory was imposed, these researches being pioneer in the study of the thermal water lily in this regard.

In terms of culture initiating starting from seeds of *N. lotus* var. *thermalis* vitrocultures, the experimental variant including liquid culture medium without filter paper support, but supplemented with 2 mg/L IAA and 2 mg/L Z (V_2L), was proven to be the most effective for germination rate (the growth compared to the control was of 30% and the percentage of germination being of 50%), demonstrating the need for a liquid column to cover the seeds, even in controlled conditions of vegetal vitrocultures. This aspect was reported for the *N. alba* species by Sumlu (S. SUMLU & al. [22]), who obtained a 51.37% rate of seed germination, but using 2 mg/L TDZ in culture medium.

The obtained results showed that the MS62 liquid medium in combination with the use of Blidar filter paper bridges (C.F. BLIDAR [28]) had a beneficial effect on the growth and development of vitoplantlets, inducing an increased rooting compared to the use of the same type of medium, but lacking solid support, and it stimulated stolons development in a higher percentage in the liquid variants without bridges. This may be associated to the fact that the thermal water lily is an aquatic plant, and in the absence of the liquid medium a robust root system develops with the purpose to reach the substrate. It was reported the success of transferring fragments rhizomes of *Nymphaea hybrid* 'James Brydon" on culture medium supplemented with activated charcoal, without hormones, for species, to stimulate the development of the root system (P. LAKSHMANAN [21]). The stolons, being part of theaquatic stem have a more intense development on culture media provided with filter paper bridges. We consider that ther is a tendency of trying to come into contact with the liquid medium to meet a solid substrate, which would have subsequently triggered the formation of new plants from their top.

Considering the extent of the caulogenesis between the two experimental series ("L" and "PB"), significant differences were noticed, in this respect, the best option being V_2L, both in terms of the number of leaflets, their size and vitality of plantlets. Studies regarding caulogenesis intensity on the *N. nouche* var. *versicolor* 'Phuean Bua' showed the efficiency of using 2.5 mg/L BAP and 0.1% activated charcoal in the culture medium (K. BODHIPADMA & al. [29]).

Remarkable differences were recorded for the gravimetric parameters which maintained the same trend, meaning that the highest values of both parameters (fresh weight and dry weight) were obtained for the *in vitro* plants grown in liquid medium supplemented with 2 mg/L IAA and 2 mg/L Z (V_2L), that showed a greater accumulation of plant biomass, compared to the "PB" series culture variant.

Conclusions

Based on those mentioned above, it can be concluded that the initiation of a *in vitro* culture starting from seeds of *N. lotus* var. *Thermalis* is possible, The optimum experimental variant for micropropagating this endengered species is a modified liquid culture medium MS62 without filter paper bridges and supplemented with 2 ml/L IAA and 2 ml/L Z. On these conditions it was achieved the highest rate of germination and caulinar multiplication. Using filter paper bridges as support for inocula ("PB" series) generated the stolons differentiation and development, stimulated rooting and the development of the *in vitro* plants, as demonstrated by the spear shaped leaves, compared to the linear form exclusively generated under hypoxia (series "L").

Since the species is in imminent danger of extinction, we recommend as main objective to continue the investigations for extending the thermal water lily phytobase (*in vitro*), as well as the establishment of an *ex vitro* nursery in order to ensure the plant material required for future ecological restauration of "Peţea stream" Natural Reserve. These results may further support the development of cost-effective conservation measures of this endengered species both *in vitro* and *in situ* (M.M. ANTOFIE [30]).

References

1. E.S. SULAIMAN, Development of sterilisation procedures and *in vitro* studies of *Nymphaea lotus*. Resume of PhD Thesis, coordinator: Mohd. Salleh, Agro-Technologie Department, Agriculture Dep., University of Putra, Malaysia, 24 p. (2004).
2. O.J. AKINJOGUNLA, A.A. ADEGOKE, I.P. UDOKANG, Antimicrobial potential of *Nymphaea lotus* (Nymphaeaceae) against wound pathogens. *Journal of Medicinal Plants Research,* 3(3): 138-141 (2009).
3. A.A. SIKDER, H.R. JISHA, R. KUDDUS, F. RUMI, M.A. KAISAR, M.A. RASHID, Evaluation of Bioactivities of *Nymphaea nouchali* (Burm. f) – the National Flower of Bangladesh. *Bangladesh Pharmaceutical Journal,* 15(1): 1-5 (2012).
4. J. ZHAO, T. LIU, L. MA, M. YAN, Z. GU, Y. HUANG, F. XU, Y. ZHAO, Antioxidant preventive Effects of Extract from *Nymphaea candida* Flower on *In vitro* Immunological Liver Injury of Rat Primary Hepatocyte Cultures. *Evidence-Based Complementary Alternative Medcine*, June, 23. doi: 10.1093/ecam/nep003; pmid: 19196740 (2011).
5. A. BOSE, M. SAHOO, S.D. RAY, *In vivo* evaluation of anti-diarrheal activity of the rhizome of *Nymphaea alba* (Nymphaeaceae). *Oriental Pharmacy Experimental*

Medicine, 12(2): 129-134. Doi 10.1007/s13596-012-9962-6 (2012).
6. A. BOSE, S. DEY RAY, M. SAHOO, Central depressant activity of ethanol extract of *Nymphaea alba* rhizome in mice. *Oriental Pharmacy and Experimental Medicine,* 13(2): 159-164. DOI: 10.1007/s/13596-013-0108-4 (2013).
7. V.M. DANCIU, Management project of "Peţea Creek" protected area from 1 Mai. *Nymphaea, Folia Naturae Bihariae,* 33: 83-102 (2006) [in Romanian].
8. V.M. DANCIU, Natural Reserve from 1 Mai in the early IIIrd millennium. *Nymphaea, Folia Naturae Bihariae,* 31: 111-131 (2004) [in Romanian].
9. T. TOFAN, Systematic abstract of vascular flora from the "Peţea Creek" - 1 Mai (jud. Bihor) nature reserve. *Nymphaea, Folia Naturae Bihariae,* 30: 85-100 (2003) [in Romanian].
10. J. TUZSON, A *Nymphaea lotus* csoport morfologiaja es rendszertani tagolodasa. *Matematikai es Termeszettudomanyi Ertesito,* Budapest, pp. 101-137 (1908) [in Hungarian].
11. T. BORSCH, K.W. HILU, J.H. WIERSEMA, C. LÖHNE, W. BARTHLOTT, V. WILDE, Phylogeny of *Nymphaea* (Nymphaeaceae): evidence from substitutions and microstructural changes in the chloroplast trnT-trnF region. *Int. J. Plant Sci.,* 168(5): 639-671 (2007).
12. P. POCZAI, K.K. MATYAS, I. SZABO, I. VARGA, J. HYVÖNEN, I. CERNÁK, A.M. GORJI, K. DECSI, J. TALLER, Genetic variability of thermal *Nymphaea* (Nymphaeaceae) populations based on ISSR markers: implications on relationships, hybridization, and conservation. *Plant Mol. Biol. Rep.,* 29: 906-918 (2011).
13. C. OLTEANU-COSMA, The thermal water lily from 1 Mai (Oradea) require protection. Nymphaea, *Folia Naturae Bihariae,* 4: 5-34, (1991) [in Romanian].
14. T. OPRIS, Encyclopedia of natural curiosities. Garamond Publisher, Bucharest, 452 p. (2000) [in Romanian].
15. L. FODORPATAKI, L. SZIGYARTO, Reproduction of plants and biotechnological applications of artificial plant multiplication. Cluj University Press, Cluj-Napoca, ISBN 978-973-610-740-5, pp. 137-143 (2008).
16. Z.O. KISS, A. BALOGH, L. FODORPATAKI, Investigation of the *in vitro* regeneration of mericlones in the Caribe variety of carnation. *International Journal of Horticultural Science,* 7(3-4): 87-89 (2001).
17. A. PĂUNESCU, Biotechnology for endangered plant conservation: a critical overview. Rom. Biotechnol. Lett., 14(1): 4095-4103 (2009).
18. M. JENKS, M. KANE, F. MAROUSKY, D. MCCONNEL, T. SHEEHAN, *In vitro* estabilishment and epiphyllous plantlet regeneration of *Nymphaea* "Daubeniana". *HortScience,* 25(12): 1664 (1990).
19. M.A. JENKS, M.E. KANE, D.B. MCCONNELL, Shoot organogenesis from petiole explants in the aquatic plant *Nymphoides indica*. *Plant Cell Tissue and Organ culture,* 63: 1-8 (2000).
20. P.R. SWINDELLS, *In vitro* reproduction of *Nymphaea*. Combined Proceedings – International Plant Propagators' Society, 40: 299-302 (1990).
21. P. LAKSHMANAN, *In vitro* estabilishment and multiplication of *Nymphaea* hybrid „James-Brydon". *Plant Cell, Tissue and Organ Culture,* 36: 145-148 (1994).
22. S. SUMLU, H.H. ATAR, K.M. KHAWAR, Breaking seed dormancy of water lily (*Nymphaea alba* L.) under *in vitro* conditions. *Biotechnology and Biotechnological Equipment,* 24: 1582-1586 (2010).
23. A. MAROSSY, Anthropogenic factors that have altered the ecological balance of the "Peţea Creek" natural reserve. *Nymphaea, Folia Naturae Bihariae,* 4: 261-272 (1976) [in Romanian].
24. V. ŞOLDEA, Peţea and the thermal water lily. Oradea University Press, p. 98 (2003) [in Romanian].
25. G. PAAL, Synthesis of hydrogeological features of thermal aquifer from Băile Felix – 1 Mai. *Nymphaea, Folia Naturae Bihariae,* 40: 83-107 (2013) [in Romanian].
26. I. COHUT, Dynamics of Oradea-Felix-1Mai hydrogeothermal system. *Nymphaea, Folia Naturae Bihariae,* 40: 109-125 (2013) [in Romanian].
27. T. MURASHIGE, F. SKOOG, A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum,* 15: 473-497 (1962).
28. C.F. BLIDAR, Evolution of *Cymbidium hybridum* protocorms grown on liquid medium "in vitro", on filter paper bridges, according to the inoculation season, pp. 213-227. In: Cachiţă, D., M., Ardelean, A., Fati, V. (eds.): Works of the XIIth National Symposium of Plant Cell and Tissue Culture "Plant cell phytopathology in vitro cultures regime", 5 June 2003 Jibou, Daya Press, Satu-Mare (2004) [in Romanian].
29. K. BODHIPADMA, S. NOICHINDA, P. WACHIRA-BONGKOTH, E. PUKPOOMIN, L. PUNNAKANTA, K. NATHALANG, (2011): *In vitro* Propagation of *Nymphaea nouchali* var. *versicolor* 'Bua Phuean'. *Environment and Natural Resources,* 9(2): 7-11.
30. M.M. ANTOFIE, Current political commitments' challenges for ex situ conservation of plant genetic resources for food and agriculture. *Analele Universităţii din Oradea, Fascicula Biologie,* 18: 157-163 (2011).