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ORIGINAL PAPER

New methods in vitroplantlets acclimatization

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Abstract

The purpose of this study was to identify a new cheap method at everyone's hand and easily applicable to improve the in vitro plantlets adaptability to the life septic environment, during the acclimatization period of micropropagation. For the Chrysanthemum and Saintpaulia ex vitro plantlets the solution which has been found and proved was spraying leaves, during the acclimatization, with deuterium depleted water (with 25 ppm deuterium), which leads to the significant evapoperspiration reduction by halving the number of stoma, by inhibiting caulogenesis and stimulating formation of more roots and also to enhance photosynthesis by increasing the synthesis of assimilated pigments. As alternative methods of deuterium depleted water spraying, by which it could enhance the synthesis of assimilator pigments or reduce the number of exvitroplantlets stoma, we recommend watering at the base of ex vitro plantlets with Pi water.

Keywords: deuterium, Pi water, acclimatization, stomata, assimilating pigments

Introduction

Deuterium (D) depleted water (DDW), contains only 25 ppm deuterium (isotope of hydrogen) versus the natural waters with a deuterium concentration of about 150 ppm [1] has been successfully used in treating human cancer, this type of water inhibate the mitotic division [2]. Our researches, regarding the effects of deuterium depleted water on the plant, began in 2001, when the team led by Prof.Univ Dr. Cachiță recorded - on natural life conditions - a plantlets growth inhibition in the first 2-3 days after placing the wheat, corn and radishes seeds for germination, on substrates wetted with DDW. Length values of shoots and their rootless were lower than those of control group, lots which was wetted with distilled water (DW), but the mentioned inhibitory effects disappeared within 5-7 days after placing them to germinate. This demonstrates the plantlets adaptability, within relatively wide range, to the medium with low deuterium concentration [3]. Similar situation was reported [4, 5, 6], in case of oat and rice germination. PETRUŞ - VANCEA et al. [7], also studied the DDW effect on *in vitro* germination of wheat, rye and triticale caryopses. Several researches in the field of using DDW to plants were also made [8, 9, 10, 11].

Pi water (PiW) production is based on the Bio Control Technologies, developed for 40 years in Japan and other countries in the world. PiW is marketed for human use and is obtained by purifying and bioenergysing drinking water with Life Energy facility, removing in this way the harmful substances from it, getting particular physical and chemical properties [12, 13].

The interest of researchers to exploit this type of water has increased enormously, as a proof is the RO 112481 1999 patent [14], for a technique that refers to the possible recovery of soil, damaged with petroleum residues, through its irrigation with PiW, achieving in this way an activation of the specific microbiota, able to destroy the oil residues and to develop the soil's normal oxide-reducing flora and fauna.

The numerous studies (biochemistry, cito-histo-anatomy and ultrastructure) of *Coleus*, *Beta* and *Petunia in vitro* cultures, made during 2001 – 2009 by RADOVET and CACHITA [15]; CACHIŢĂ et al. [16]; PETRUŞ and CACHITA [17] and PETRUŞ-VANCEA et al. [18,

19] have clearly demonstrated the role of both DDW and PiW in preventing and annihilating vitroplantlets hyperhydricity.

In the current context of bio-economy and eco-economy [20], we intend to improve the qualitative and quantitative *in vitro* cultured plantlets acclimatization to septic medium, by cheap, efficient and handy methods, using PiW and DDW.

Material and method

Plant materials used were Chrysanthemum (*Chrysanthemum morifolium* Ramat var. Lamet) and African violet (*Saintpaulia ionantha* L.) *ex vitro* plantlets (Table 1) obtained from the vitrocultures as described by MURASHIGE – SKOOG [21] culture medium, modified in this study. For acclimatization, *Chrysanthemum* exvitroplantlets with 3 to 4 cm size (at 35 days of vitroculture) and *Saintpaulia* with size of 1 to 1.5 cm were selected, which at the time of their removal from *vitro* were aged 90 days old. Prior to planting them in the substrate, the solidified medium was removed from the vitroplantlets rootlets, after which they were placed in perlite, humidified with 250 ml of distilled water (control group) and PiW or DDW with only 25 ppm D.

Table 1. Experimental protocol.

Culture medium	Cods	*Ex vitro* sprinkling type		Exvitroculture conditions	Biometrizations (at 30 days after acclimatization period start)
- *Murashige-Skoog* (1962), solidified - without regulators and amino acids	V_0 *control*	Foliar	*DW*	- exvitroculture illuminated with white fluorescent tubes, with 1700 lx intensity; - 16 h light/24 h photoperiod; - varied temperature between 20 – 23 ºC; - substratum: perlite.	-survival percent of acclimatization; -density, length and width of stomata; - assimilating pigments; - grown indexes.
		Basal	*DW*		
	V_1	Foliar	PiW		
		Basal	*DW*		
	V_2	Foliar	*DW*		
		Basal	PiW		
	V_3	Foliar	DDW		
		Basal	*DW*		
	V_4	Foliar	*DW*		
		Basal	DDW		

Note: DW – distilled water; DDW – deuterium depleted water; PiW – Pi water.

Vitroplantlet acclimatization to septic environment

Vitroplantlets acclimatization to a septic environment was made in plastic boxes, rigid, made - in the basal part – from a plastic tray with dimensions of 30/20/5 cm, covered with a transparent, colorless, vaulted two parts sliding, plastic cover. Each experimental group had an autonomy regarding the treatment organization, the root substrate being covered with aluminum foil and exvitroplantlets stemlets being pulled through some small holes with a diameter of 0.5 cm for chrysanthemums and 1 cm for African violets.In the first 7 days of acclimatization, watering crops, with different types of water, was achieved either by spraying, with 10 ml water/plot, either by sprinkling on each exvitroplantlets (with 5 ml of water/plant), every two days. In the next 21 days, to avoid excessive moistening of the substrate, wetting it with different types of water, depending on the experimental variant was practiced once a week by spraying, again with 10 ml/variant plus the application of each 10 ml, to each plantlets base, throughout acclimation experiment each version received 5 ml of water applied foliar, all the water given to each exvitroplantlets was 65 ml. Spraying was done with a spray purchased from trade.

Stomas Examination and Biometrisation

Stomas examination and biometrisation was made microscopically, on film casts consisting of collodium, stamping the top of epidermal foliar limbs to the *Chrysanthemum* (which are amphystomatic limbs) or low epidermis to *Saintpaulia* (which are hypostomatic) leaflet, resulted in the acclimatization period. The obtained preparations were examined at the optical microscope Leitz, Webster M label. The evaluation of the stomata number, per

microscopic field, was made by the 40X lens and with a 10X (400X) eyepiece. The micrometric index was determined after the ANDREI and PARASCHIVOIU [22] method.

Assimilating pigments, respectively chlorophylls a, b and carotenoid pigments were determined from leafs, by extracting them in N, N-dimethylformamide (DMF) 99.9%, according to the method developed by MORAN and PORATH [23] and the quantitative values were obtained by using the absorption coefficients of WELBURN [24]. Total assimilating pigments was calculated by summing chlorophyll a and b and carotenoid pigments extracted from under analysis plant material. Pigment extractions were made from fresh plant material.

Biometrical grown indexes dates were mathematically [the values recorded at 30 days from *ex vitro* plantlets placing in the septic medium, concordant to the experimental variants $V_1 – V_4$, were related to the homologues dates], biometrisated to the control lot (V_0), *ex vitro* rooted in aerial part humidification conditions or in substratum watered with distillate water, these being considered as 100%. All values was statistically (*t* test) processed.

Results and discussions

Post-acclimatization survival percent of *Chrysanthemum* and *Saintpaulia* exvitroplantlet lots, at 30 days after they were transferred *ex vitro*, being treated with various types of water, according to $V_0 – V_4$ experimental variants were 100%.

Growth indexes. Regarding rhysogenesis, expressed by rootlets length, both to the *Chrysanthemum* and to the *Saintpaulia*, all four treatments caused the inhibition process, except rootlets of African violets plantlets which have been wetted to the root with PiW (V_2) and the leafs were wetted with DDW (V_3), lots on which were recorded slight increases of up to 15% of control, but statistically non-significant, situation maintained to this plantlets in case of rootlets number too, but increases in this latter parameter is highly statistically significant, with values expressed as a percentage of 50% (Table 2).

Caulogenesis was inhibited in *Chrysanthemum* by the foliar application of PiW (V_1) and by the root application of DDW (V_4), lots which were noticed larger or smaller reduction, to all monitored parameters, they were significant or non significant from statistical point of view (Table 2). Caulogenesis stimulation at the *Chrysanthemum* was achieved through the root application of PiW (V_2), but only if the number of nodes or stemlets where the levels were highly statistically significant, and in case of DDW (V_3) foliar application, but increases was highly statistically significant only in the number of branches (Table 2). At African violets, the group with PiW (V_1) sprayed leaflets, did not form new propagules, but the total number of leaflets, with an average of 7.25 individs/seedlings, was higher by 7%, to control (V_0), which was represented by the *ex vitro* plantlets [both foliar and basal] watered with AD, the difference between the average of the two groups, in regard to this parameter, being statistically significant. If rhysogenesis knew increases by applying PiW (V_2), to the *Saintpaulia* exvitroplantlets, this treatment had not the same effect on caulogenesis in this species. In a similar experiment, but made in natural conditions, *Tradescantia* cuttings was studied [25]. The spraying effect – during 30 days - on the foliar limbs of *Tradescantia* sp. cuttings leafs, and on the rooting substratum, made with PiW or DDW (with 25 ppm D), concerning rhysogenesis and plantlets growth index, or about stomata apparatus, was identified at the level of inferior epidermis of foliar limb. The authors concluded that Pi water proved to have a stimulator effect on *Tradescantia* cuttings organogenesis, by foliar or radicular application. The deuterium depleted water increased only the rhisogenesis, by foliar spraying, comparatively to the control lot (foliar limb and basal cuttings sprinkled with distilled water - DW), which stomas were numerous at the level of foliar limbs of the plantlets sprinkled, to the base, with Pi water and foliar with distillate water.

Table 2. Grown indexes of *Chrysanthemum morifolium* Ramat var. *Lamet* and *Saintpaulia Ionantha* L. exvitroplantlets in a *30 days* of acclimatization, proceeding from follow experimental type: V_0 – exvitroplantlets foliar sprinkled and watered to the base with *distillate water* (control); V_1 - exvitroplantlets foliar sprinkled with *Pi water* and watered to the base with *distillate water*; V_2 - exvitroplantlets foliar sprinkled with *distillate water* and watered to the base with *Pi water;* V_3 - exvitroplantlets foliar sprinkled with *deuterium depleted water, with 25 ppm D* and watered to the base with *distillate water*; V_4 - exvitroplantlets foliar sprinkled with *distillate water* and watered to the base with *deuterium depleted water, with 25 ppm D*. V_1 – V_4 values have been reported toward the ones registered at the control plants (V_0), these being considered as 100%.

Chrysanthemum morifolium* Ramat var. *Lamet

Types	*Eexvitroplantlets size*		Rhysogenesis				Caulogenesis							
			Rootlets length		*Rootlets number*		*Stemlet length*		*Nods number*		*Branches number*		*Stemlets number*	
	average (cm) ± standard deviation	%	average (cm) ± standard deviation	%	average (no.) ± standard deviation	%	average (cm) ± standard deviation	%	average (no.) ± standard deviation	%	average (no.) ± standard deviation	%	average (no.) ± standard deviation	%
V_0	**14.69 ± 1.45**	**100**	**9.38 ± 1.32**	**100**	**6.25 ± 0.15**	**100**	**5.31 ± 0.15**	**100**	**11.88 ± 0.12**	**100**	**0.38 ± 0.17**	**100**	**1.00 ± 0.00**	**100**
V_1	9.25 ± 0.23 *	63	4.38 ± 0.17 **	47	6.25 ± 0.15 ns	100	4.88 ± 0.12 ns	92	8.88 ± 0.12 **	75	0.25 ± 0.15 ns	66	1.00 ± 0.00 ns	100
V_2	13.13 ± 0.23 *	89	8.13 ± 0.12 *	87	9.13 ± 0.12 **	146	5.00 ± 0.15 ns	94	13.13 ± 0.12 *	111	0.25 ± 0.15 ns	66	1.38 ± 0.17 **	138
V_3	12.56 ± 0.47 *	86	7.38 ± 0.47 **	79	9.75 ± 0.15 **	156	5.3 ± 0.09 ns	100	13.00 ± 0.35 ns	109	0.13 ± 0.12 **	34	1.13 ± 0.12 ns	113
V_4	9.81 ± 0.59 **	67	5.00 ± 0.47 **	53	4.25 ± 0.15 **	68	4.81 ± 0.22 ns	91	11.25 ± 0.15 ns	95	0.00 ± 0.00 -	0	1.00 ± 0.00 ns	100

***Saintpaulia ionantha* L.**

Types	Rhysogenesis				Caulogenesis											
	Rootlets length		*Rootlets number*		*Propagules number*		*Leaflets total number*		*Leaflets with 0.0-0.4 cm diameter*		*Leaflets with 0.5-0.9 cm diameter*		*Leaflets with 1.0-1.4 cm diameter*		*Leaflets with 1.5-1.9 cm diameter*	
	average (cm) ± standard deviation	%	average (no.) ± standard deviation	%	average (no.) ± standard deviation	%	average (no.) ± standard deviation	%	average (no.) ± standard deviation	%	average (no.) ± standard deviation	%	average (no.) ± standard deviation	%	average (no.) ± standard deviation	%
V_0	**2.00 ± 0.00**	**100**	**4.00 ± 0.00**	**100**	**1.00 ± 0.00**	**100**	**6.8 ± 0.08**	**100**	**2.04 ± 0.04**	**100**	**1.42 ± 0.10**	**100**	**2.63 ± 0.10**	**100**	**0.71 ± 0.09**	**100**
V_1	1.42 ± 0.10 **	71	3.54 ± 0.10 **	89	1.00 ± 0.00 ns	100	7.25 ± 0.09 *	107	2.58 ± 0.10 **	126	2.17 ± 0.08 **	153	1.29 ± 0.9 **	49	1.21 ± 0.08 **	170
V_2	2.15 ± 0.04 ns	108	6.00 ± 0.00 **	150	1.00 ± 0.00 ns	100	6.78 ± 0.08 ns	100	1.41 ± 0.09 **	69	2.00 ± 0.00 **	141	1.70 ± 0.09 **	65	1.67 ± 0.09 **	235
V_3	2.29 ± 0.09 ns	115	6.00 ± 0.00 **	150	1.00 ± 0.00 ns	100	6.54 ± 0.13 ns	96	1.29 ± 0.09 **	63	2.00 ± 0.00 **	141	2.25 ± 0.09 **	86	1.00 ± 0.00 **	141
V_4	1.29 ± 0.05 **	65	2.67 ± 0.10 **	67	1.00 ± 0.00 ns	100	7.33 ± 0.11 ns	108	1.00 ± 0.00 **	49	2.71 ± 0.09 **	191	1.63 ± 0.10 **	62	2.00 ± 0.00 **	282

Note: ns – non significant, * - significantly ($p < 0.05$), ** - very significantly ($p < 0.01$).

For *Cymbidium* protocorms [26], cultivated in liquid medium, the presence of DDW (with only 25 ppm D) led to drastic reduction of the multiplication rate and of the protocorms growth, and at the *Petunia* vitroplantlets the rhysogenesis and caulogenesis was obviously inhibited by the presence of DDW in MS culture medium composition, in both deuterium concentration of 25 ppm and 87.5 ppm, used as replacement for distilled water. This has confirmed the inhibitory effect of such water on growth and plant cell differentiation.

Stomata measurement. At 30 day after starting the acclimatization process, at both species, the stomatas (st.) was closed when measurements were made. At the *Chrysanthemum* (amphystomatics) the stomata density of the upper epidermis was 4 st/microscopic field, except that when the leaves were sprayed with DDW (V_3), that were encountered, on average 2 st./field, highly statistically significant reductions compared to control (both foliar and basal exvitroplanttlets treated with distilled water - V_0), percentage being expressed by values of 50% (Table 3). Because *Saintpaulia* leaflets are hypostomatics, to study their stomas, only lower epidermis amprentation were made. There were also chosen leaflets from the top of the rosette, which were the youngest new *ex vitro* formed. The highest stomata density - 16 st./field - was recorded in foliar and basal treated leaf with DW, belonging to the control group (V_0) and the lowest frequency has been marked to exvitroplantlet leaflets treated foliar with DDW (V_3), this marked a very significant reduction - the percentage of 44% - to control V_0 (Table 3). Stomata dimensions (width and length) belonging to both of *Chrysanthemum* and *Saintpaulia* exvitroplantles, which have different types of water treatment, had similar values - 37.7/18.9 µm - except those of the control, that presented the smaller stomatas with 28.3/18.9 µm, signaling statistically, very significant increases to it, expressed as a percentage by value of 33%, in all four experimental variants (V_1 - V_4).

We must mention that, in both species, there were not observed changes in densities of tector hair depending on the treatment.

Table 3. Dates of stomata biometric measurements done at the level of *Crhysanthemum* and *Saintpaulia* exvitroplantlets leaflets, in a *30 days* of acclimatization proceeding from follow experimental type: V_0 – exvitroplantlets foliar sprinkled and watered to the base with *distillate water* (control); V_1 - exvitroplantlets foliar sprinkled with *Pi water* and watered to the base with *distillate water*; V_2 - exvitroplantlets foliar sprinkled with *distillate water* and watered to the base with *Pi water;* V_3 - exvitroplantlets foliar sprinkled with *deuterium depleted water* and watered to the base with *distillate water*; V_4 - exvitroplantlets foliar sprinkled with *distillate water* and watered to the base with *deuterium depleted water*. V_1 – V_4 values have been reported toward the ones registered at the control plants (V_0), these being considered as 100%.

Species	Types	Stomata density		Length of stomata cells		Width of stomata cell	
		average (no.) ± standard deviation	%	average (µm) ± standard deviation	%	average (µm) ± standard deviation	%
Chrysanthemum morifolium Ramat var. *Lamet*	**V_0**	**4±0.7**	**100**	**28.3±1.34**	**100**	**18.9±1.3**	**100**
	V_1	4±1.00	100	37.7±1.90 **	133	18.9±0.00	100
	V_2	4±0.00	100	37.7±1.90 **	133	18.9±1.80	100
	V_3	2±0.00 **	50	37.7±0.00 **	133	18.9±0.00	100
	V_4	4±0.00	100	37.7±0.00 **	133	24.5±1.90	130
Saintpaulia ionantha L.	**V_0**	**16±6.3**	**100**	**28.3±1.45**	**100**	**28.3±1.5**	**100**
	V_1	13±0.50 *	81	37.7±0.69 **	133	18.9±0.00 **	67
	V_2	13±0.40 *	81	37.7±0.98 **	133	18.9±0.00 **	67
	V_3	9±0.820 **	56	37.7±1.20 **	133	18.9±2.00 **	67
	V_4	13±0.60 *	81	37.7±1.39 **	133	18.9±2.80 **	67

Note: ns – non significant, * - significantly ($p < 0.05$), ** - very significantly ($p < 0.01$).

Assimilating pigments. After 30 days of their transferr in soil, the total quantity of assimilating pigments (summing chlorophyll a and b with carotenoid pigments) was statistically significant, compared with the control, to all groups treated with different types of waters, with the exception of radicular watered with PiW (V_2), which had for Chrysanthemums a slight decrease, in statistically terms, but very significant for African violets (Table 4). At Chrysanthemums, wetting the leaf with DDW led to the biggest incentives of assimilating pigment synthesis, with 74% higher to the control, and at *Saintpaulia*, DDW also stimulated the synthesis of assimilating pigments, but especially in the case of exvitroplantlets basal sprinkling (Table 4).

To *Coleus* vitrocultures - if over the initial solid medium was placed a second layer of PiW, or PiW combined with glucose - the highest values of chlorophyll pigments were found at the variant containing supernatant PiW plus glucose [24]. However, to decrease caulogenesis we particularly recommend to spray the *ex vitro* transferred plantlet leaflets with deuterium depleted water (with 25 ppm D), in favor of intensifying the rhyzogenesis, to decrease the number of stomata on the surface of foliar leaflets, and stimulating the synthesis of assimilating pigments, all these factors being particularly important in improving the adaptability of exvitroplantlets to septic conditions, leading to obtain maximum yield of young vigorous plants.

Table 4. Total assimilating pigments quantity extracts from the leaflets of exvitroplantlets (prelevated in a *30 days* of acclimatization) at *Chrysanthemum morifolium* Ramat var. *Lamet* and *Saintpaulia ionantha* L. proceeding from follow experimental type: V_0 – exvitroplantlets foliar sprinkled and watered to the base with *distillate water* (control); V_1 - exvitroplantlets foliar sprinkled with *Pi water* and watered to the base with *distillate water*; V_2 - exvitroplantlets foliar sprinkled with *distillate water* and watered to the base with *Pi water;* V_3 - exvitroplantlets foliar sprinkled with *deuterium depleted water, with 25 ppm D* and watered to the base with *distillate water*; V_4 - exvitroplantlets foliar sprinkled with *distillate water* and watered to the base with *deuterium depleted water, with 25 ppm D*. V_1 – V_4 values have been reported toward the ones registered at the control plants (V_0), these being considered as 100%.

Species	Types	Total assimilating pigments	
		average (μg/gSP) ± standard deviation	%
Chrysanthemum morifolium Ramat var. *Lamet*	**V_0**	**16.4978±0.0090**	**100**
	V_1	21.8751±0.0100**	132
	V_2	16.3588±0.0110ns	99
	V_3	28.7638±0.0905**	174
	V_4	25.3475±0.0220**	153
Saintpaulia ionantha L.	**V_0**	**15.1123±0.1100**	**100**
	V_1	18.1048±0.0608**	120
	V_2	13.6531±0.1528**	90
	V_3	20.8143±0.0120**	138
	V_4	21.6365±0.0900**	143

Note: ns – non significant, * - significantly ($p < 0.05$), ** - very significantly ($p < 0.01$).

Conclusions

1. Treating *Chrysanthemum* exvitroplantlets with Pi water, during their acclimatization to *ex vitro*, exercised stimulatory effects over the plant growth and assimilating pigments synthesis only when it was applied on a leaf, by spaying. Sprinkling basal plantlets with Pi water did not show major influence on the process of *Chrysanthemums* exvitroplantlets adaptation to septic medium.
2. Treating *Chrysanthemum* exvitroplantlets with deuterium depleted water (with 25 ppm D) generated different reactions, depending on the kind of it application. Thus, foliar spraying the exvitroplantlets with deuterium depleted water, while the substrate was sprinkled with

distilled water, caused stimulating processes of organogenesis, but especially led to halving the number of stomata in the foliar limbs. Instead, spraying leaflets with distilled water while wetting the substrate with deuterium depleted water, inhibited exvitroplantlets growth, particularly the rhysogenesis, but both treatments have increased synthesis of assimilating pigments.

3. In the case of *Saintpaulia* exvitroplantlets aclimatization, the foliar treatment with Pi water exercized an inhibitory effect of rhysogenesis, but a caulogenesis intensification; the application of such water at the basal level exercised reverse effects.
4. Spraying *Saintpaulia* exvitroplantlets with deuterium depleted water (with 25 ppm D) caused an increase of rhyzogenesis, the total number of leaflets being identical to that recorded at the control group, and reduced the number of stomata on the foliar limbs; plantlet basal applications of this water determined to the exvitroplantlets caused a strong inhibition of rootlets formation and of pigment synthesis, stimulating only the growth of leaflets with large diameter and helping to reduce the number of stomata.

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