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IN VITRO PRESERVATION OF POTATO SHOOT CULTURES

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Abstract: The conservation of higher plant tissues and organs is based on a pronounced reduction of the growth rate, achieved by manipulations of the culture conditions and of the nutrient media. In order to achieve a higher efficiency of preservation, we applied a combination of different limiting factors like the simultaneous use of hypoxia and a modified chemical composition of the nutrient medium. This paper presents two preservation approaches, reduced-growth storage of nodal cuttings and long-term storage by vitrification of apical shoot tips. In vitro grown potato (*Solanum tuberosum* L. cv. Mureşan) plantlets were used in the present study. The preservation period of potato shoots was extended from one month to twelve months by addition of growth inhibitors like abscisic acid (ABA), to the Murashige-Skoog nutrient medium and a paraffin oil overlay. The preservation of the shoots was achieved under two different conditions of illumination and temperature: under a 16 hours light photoperiod at 25±1°C, and in continuous darkness at low temperature (4±1°C). The performed studies led to the conclusion that the reduced-growth was effectiv induced by the combined action of ABA and the hypoxic regime. The best combination treatments for a moderately-term (one year) preservation of nodal cuttings consists of the addition of higher amounts (40 mg/l) of ABA in the culture medium, a paraffin oil layer and storage at 25°C with a photoperiod of 16/24 h or in darkness at 4°C. Cryopreservation was realised using a modified vitrification technique. The cryoprotective treatment with glycerol and sucrose at 25°C for 10 minutes followed by treatment with a plant vitrification solution (PVS2) induced freezing tolerance of potato apical shoot tips. Vitrified apical shoot tips resumed growth within 4 - 5 weeks after thawing and plating on MS medium supplemented with NAA 0.5 mg/l, Z 1 mg/l and GA3 0.5 mg/l.

Introduction

Reduced-growth storage has been widely used for maintain differentiated cultures. The reduced-growth storage methods can be divided into following types: the reduction of incubation temperature, the modification of culture media, the combination of the two methods and the modification of the gaseous environment [18].

Potato plants were preserved on medium containing growth retardants as maleic acid and N-dimethylaminosuccinamic acid, cinnamic acid or sucrose in high concentrations [7,20]. Nodal cuttings and calus were preserved on media supplemented with osmotic active compounds and storage at reduced temperatures [9,14,15]. Alteration of the gaseous composition in the culture vessels by using mineral oil was used to induce reduced-growth of plant germplasm [8,6,3,4,5,11,12].

This paper presents two preservation approaches, reduced-growth storage of nodal cuttings and long-term storage by vitrification of apical shoot tips.

Materials and Methods

In vitro grown potato (*Solanum tuberosum* L. cv. Mureşan) plantlets were used in the present study. For the reduced-growth (slow-growth) storage method, nodal cuttings and for cryopreservation by vitrification apical shoot tips were used. Stock cultures were maintained at 25±1°C on Murashige-Skoog [16] basal medium without phytohormones under white fluorescent light with 16 h photoperiod and 39.06 $\mu E.m^{-2}.s^{-1}$. The sterile paraffin oil overlay (2 cm) has been applied of nodal cuttings after three days following their subculturing. The used abscisic acid (ABA) (as the main inhibitor hormone in higher plants) concentrations were 10 mg/l, 20 mg/l, 30 mg/l, 40 mg/l and 50 mg/l. The medium composition in different stages of

preservation is presented in Table 1. The nodal cuttings were stored in light at 25±1°C and in darkness at 4±1°C for one year. At the end of the preservation period biometric analysis and phenotypic characters of recovered plants were studied.

The complete vitrification procedure involves: a) loading of the cells with a cryoprotective mixture containing 2 M glycerol and 0.4 M sucrose; b) dehydration of the cells by exposure to a concentrated vitrification solution PVS2 [19]; c) freezing; d) thawing and e) transffering the tissue to recovery medium. The apical shoot tips were placed after loading (10 minutes) on aluminium strips (0.6 cm x 1.5 cm) in a drop (4 µl) of vitrification solution PVS2 for other 10 minutes. The strips were immersed directly into liquid nitrogen (LN) and held for 24 h. Shoot tips were thawed rapidly. After thawing samples were plated in Petri dishes containing an MS semisolid (3.5 g/l agar) medium. Control shoot tips were exposed to the PVS2 solution as described above and then were plated on the same medium without freezing. Recovery rate expressed in percentage of treated shoot tips was estimated 45 days after plating by counting the shoot tips that had survived. Three replicates of 25 shoot tips were used for each experiment.

Table 1: Composition of culture medium used in different stages of preservation.

Cod of the culture medium	Medium composition (mg/l) pH-5.7	**Preservation stages**
V_0	MS (control – without growth regulators)	- plant multiplication - plant recovery following preservation
V_1	MS + ANA 0.5 + BA 1 + GA3 0.5	
V_2	MS + ANA 0.5 + Z 1 + GA3 0.5	
V_3	MS + AIA 0.5 + BA 1 + GA3 0.5	- plant recovery post-storage
V_4	MS + AIA 0.5 + Z 1 + GA3 0.5	
V_5	MS + AIB 0.5 + BA 1 + GA3 0.5	
V_6	MS + AIB 0.5 + Z 1+ GA3 0.5	

Results and Discussions

Slow-growth storage

After one year storage in the mentioned conditions the nodal cuttings were subcultured on fresh medium with various hormone balance (Table 1) for regeneration. In order to find the ABA concentrations with an inhibitory effect without affecting the plant viability, different ABA concentrations were tested for two thermic conditions of the preservation. The combined effect of ABA and the hypoxic condition, induced by the paraffin oil, on the survival and regenerative capacity of nodal cuttings is shown in Table 2.

Concerning the influence of different amounts of abscisic acid on the subsequent developmental potency of the nodal cuttings it is to observe the progressive increase of the regenerative capacity of the nodal cuttings with the increase of ABA concentration (Table 2). An important aspect that should be mentioned is the fact that in both tested storage temperatures, the regeneration rate was affected by the ABA concentration in the culture medium. As Table 2 shows the highest regeneration rate (65%) after one year storage was achieved at the nodal cuttings stored at 25ºC on medium with 40 mg/l ABA. Concerning the influence of the storage temperatures higher regeneration rates 25% (50 mg/l ABA) were registered when the temperature was 4ºC in comparison with 48% (50 mg/l ABA) when the temperature was 25ºC.

The nodal cuttings stored at 4ºC (after one year storage) shown an increase of the recovery rate with the increase of the ABA concentration from 7% in the case of 10 mg/l ABA to 25% at 50 mg/l ABA in the storage medium. Our results showed the inhibitory effect of ABA on the nodal potato cuttings stored for one year in hypoxic conditions without lose of plant viability.

Table 2: Effect of abscisic acid and hypoxic regime on the regenerative capacity of nodal cuttings following different storage periods.

Storage temperatures	Storage period (months)	Plant regeneration (%)				
		Abscisic acid (mg/l)				
		10	20	30	40	50
25±1ºC	3	32.6±5.5*	58.0±3.0	55.3±4.5	66.6±6.5	80.3±7.5
	6	20.6±4.0	45.0±4.5	50.0±2.0	54.0±4.5	72.0±4.5
	12	12.0±2.0	30.3±2.5	36.6±5.6	65.3±5.0	48.6±5.6
4±1ºC	3	21.0±3.6	33.3±4.0	34.0±6.2	38.3±5.5	41.0±6.2
	6	12.0±2.5	26.3±4.0	30.0±3.0	32.6±7.7	33.6±6.0
	12	7.3±3.0	17.3±5.0	19.6±7.3	21.3±6.4	25.6±5.8

Note: *standard deviation

In order to appreciate the influence of a growth inhibitor, it has to be taken into account the fact that several inhibitors applied in very small amounts will stimulate the developmental processes, and the inhibitory effects appear only at higher concentrations.

Following the results of the experiments described above regarding the influence on ABA on the slow-growth of potato nodal cuttings, we also tested the effect of hormone balance in the recovery culture medium. For this experiment we used nodal cuttings which were stored of medium with 40 mg/l ABA. The effect of phytohormone composition on the recovery and development of nodal cuttings after one year storage depending on the storage temperatures is shown in Fig. 1-3.

These data shows that the lenght of plants regenerated from stored nodal cuttings was higher in comparison with the control plants (V_0). It should be mentioned that the lowest values regarding the plant lenght were 3.8 cm for a storage temperature of 25ºC and 3.4 cm for a storage temperature of 4ºC (Fig. 1). The highest lenght of plants was 7 cm when the cuttings were stored at 25ºC and respectively 8.15 cm when the storage temperature was 4ºC in the case of MS medium supplemented with NAA 0.5 mg/l, BA 1 mg/l and GA3 0.5 mg/l (V_1). Concerning the root number, after two months from the subculturing of the nodal cuttings on culture medium with various hormone balances (Table 1), differences were found as function of the storage temperature (Fig. 2). The highest number of roots (9) was found on MS + IAA 0.5 mg/l + Z 1 mg/l + GA3 0.5 mg/l (V_4) in case of cuttings stored at 25ºC and 8.5 on MS + NAA 0,5 mg/l + Z 1 mg/l + GA3 0.5 mg/l (V_2) in case of nodal cuttings stored at 4ºC. The lowest number of roots (3.5) were registered in case of cuttings stored at 4ºC and subcultured on MS + IBA 0.5 mg/l + BA 1 mg/l + GA3 0.5 mg/l (V_5).

The effect of ABA on the root lenght is showed in Fig. 3. As Fig. 3 shows except the control plants were the root lenght was 4 cm at 25ºC and 3 cm at 4ºC the other culture medium variants ($V_1 – V_6$) showed higher values regarding the root lenght. In the case of MS + NAA 0.5 mg/l + Z 1 mg/l + GA3 0.5 mg/l medium (V_2) an average of 6.75 cm root lenght (25ºC) and 6.25 cm (4ºC) were registered. It is worth mentioning that it was possible to establish a significant positive correlation between the storage temperature (25ºC) and the composition of the recovery medium: V_0 (MS control) ($r = 0.99$) and V_3 (MS + IAA 0.5 mg/l + BA 1 mg/l + GA3 0.5 mg/l) ($r = 0.99$). Stored nodal cuttings are represented in Fig. 4 a, b, c, and regenerated whole plantlets from stored nodal cuttings on culture medium with various growth regulators are represented in Fig. 5 a, b.

When the storage temperature was 4ºC a significant positive correlation was established between the number of roots and their lenght on the following variants: V_2 (MS + NAA 0.5 mg/l + Z 1 mg/l + GA3 0.5 mg/l) ($r = 0.97$) and V_5 (MS + IBA 0.5 mg/l + BA 1 mg/l + GA3 0.5 mg/l) ($r = 0.99$). The regenerated plants did not presents phenotypic modification after storage

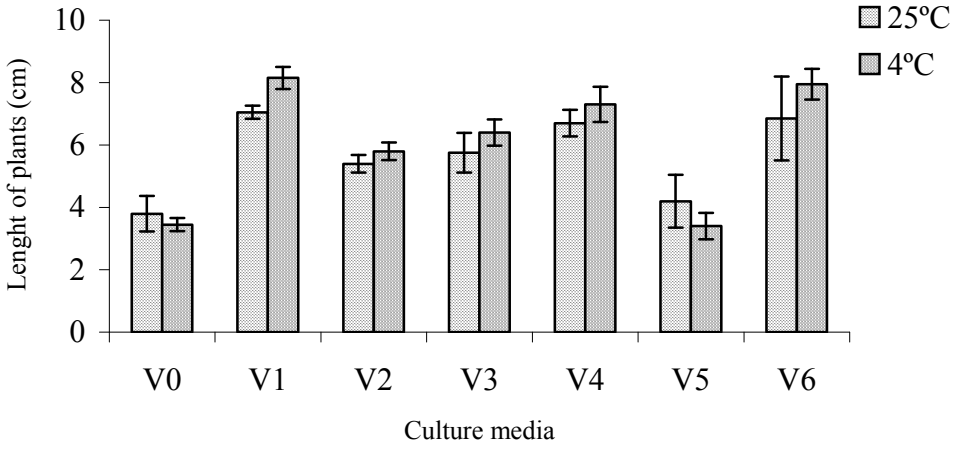

Fig. 1.

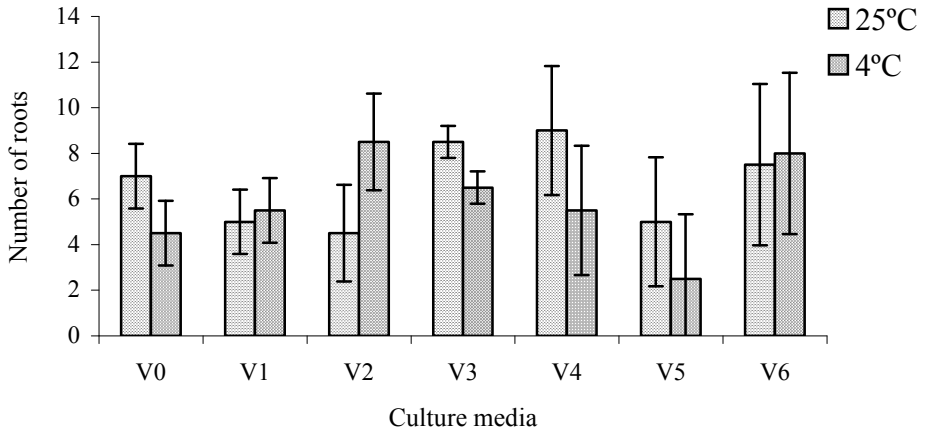

Fig. 2

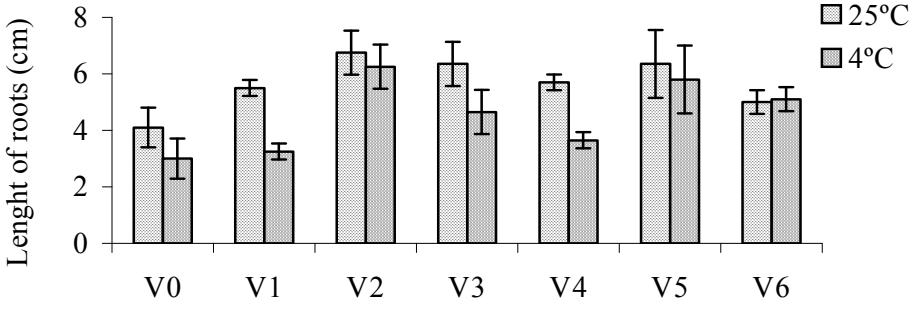

Fig. 3

Fig. 1-3: Effect of storage conditions on the plant development on medium with different hormone balance.V0=MS (control); V1=MS+NAA 0.5 mg/l+BA 1 mg/l+GA3 0.5 mg/l; V2=MS+NAA 0.5 mg/l+Z 1 mg/l+GA3 0.5 mg/l; V3=MS+IAA 0.5 mg/l+BA 1 mg/l+GA3 0.5 mg/l; V4=MS+IAA 0.5 mg/l+Z 1 mg/l+GA3 0.5 mg/l; V5=MS+IBA 0.5 mg/l+BA 1 mg/l+GA3 0.5 mg/l; V6=MS+IBA 0.5 mg/l+Z 1 mg/l+GA3 0.5 mg/l. Vertical bars represents standard deviation.

Fig. 4: *In vitro* potato plantlets. a) nodal cuttings donor plants; b) nodal cuttings on medium with ABA and under paraffin oil layer; c) regenerated plantlets from stored nodal cuttings.

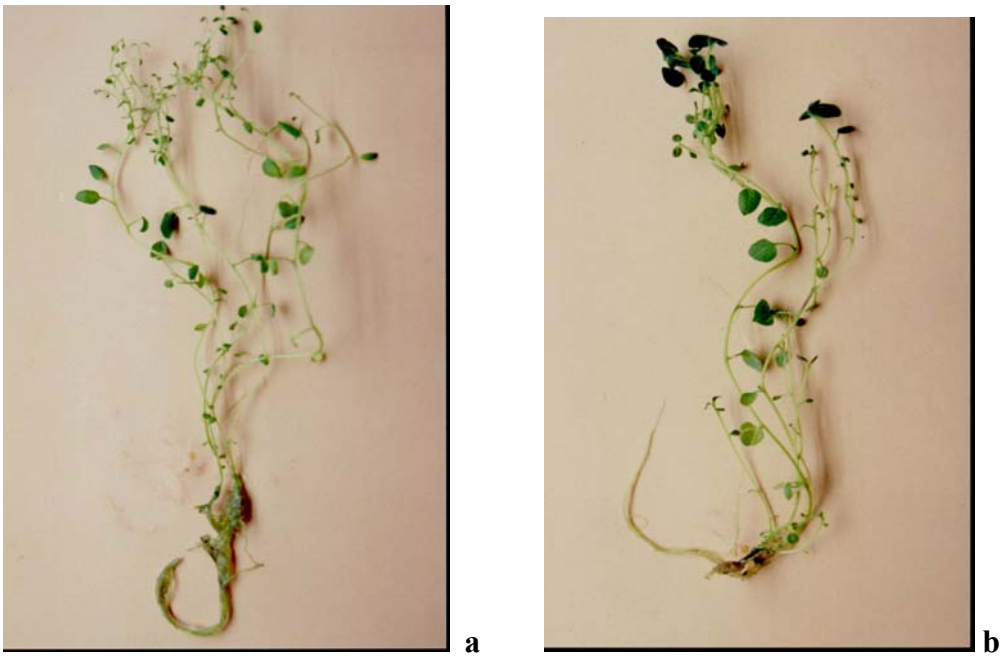

Fig. 5: Regenerated potato plantlets from stored nodal cuttings on MS medium supplemented with NAA 0.5 mg/l, BA 1 mg/l and GA3 0.5 mg/l (a) and with IBA 0.5 mg/l, Z 1 mg/l and GA3 0.5 mg/l.

ABA increases the stress tolerance of plants conferring an enhanced capacity of survival under less favorable conditions, but on the other hand it induces senescence and metabolic

inhibition, so its use is recommended only during latent physiological state of the *in vitro* plant cultures [10,17].

Cryopreservation by vitrification

Vitrified apical shoot tips resumed growth within 4-5 weeks after thawing and plating on MS medium supplemented with NAA 0.5 mg/l, Z 1 mg/l and GA3 0.5 mg/l. To increase the applicability of this vitrification method, the shoot tips were loaded and treated with diluted PVS2 solution (20%, 40%, 60%, 80% of the stock PVS2) at 25°C prior to plunge in liquid nitrogen. As shown in Table 3, even the 80% PVS2 produced high levels of survival (44%). The highest survival (62%) rate was registrated when PVS2 100% were used. Unfrozen control shoot tips were not affected by the high concentration of PVS2.

Table 3: Survival of apical shoot tips cooled to -196°C after dehydration with PVS2.

Concentration of PVS2 (%)	Control apical shoot tips (unfrozen) (%)	Vitrified apical shoot tips after freezing (%)
20	91.33±3.21*	0
40	94.00±3.60	7.66±3.51
60	89.67±6.65	23.00±7.54
80	80.66±4.04	44.33±6.02
100	80.00±8.00	62.00±6.24

Note: *standard deviation

Concerning the phenotypic modification of plants after cryopreservation it was demonstrated that the process of cryopreservation of shoot-tips has less effect on the plants phenotype [1,2,13].

Conclusions

The performed studies led to the conclusion that the reduced-growth was effectiv induced by the combined action of ABA and the hypoxic regime.

The best combination treatments for a moderately-term (one year) preservation of nodal cuttings consists of the addition of higher amounts of ABA in the culture medium, a paraffin oil layer and storage at 25°C with a photoperiod of 16 h/ 24 h or in darkness at 4°C. For a high recovery rate post-storage it is recommended that the subculture should be made on MS medium supplemented with NAA 0.5 mg/l + BA 1 mg/l + GA3 0.5 mg/l (V_1) and MS supplemented with IAA 0.5% mg/l + BA 1 mg/l + GA3 0.5 mg/l (V_3).

The cryoprotective treatment with glycerol and sucrose at 25°C for 10 minutes followed by treatment with PVS2 induced freezing tolerance of potato apical shoot tips and ensured their survival after freezing in liquid nitrogen.

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CONSERVAREA IN VITRO A MINIBUTAŞILOR DE CARTOF

(Rezumat)

Conservarea prin creştere lentă. Culturi *in vitro* constând din minibutaşi binodali de cartof au fost conservați în condiții de hipoxie şi pe medii conținând inhibitori de creştere, respectiv acid abscisic. Aceste culturi au fost menținute timp de un an, fie în regim de alternanță a luminii (fotoperioadă de 16 ore lumină/ 24 ore) cu întunericul, la temperatura de 25°C, fie în regim de întuneric continuu, la temperatura de 4°C. După încheierea duratei de conservare din minibutaşii conservați au regenerat plantule care nu au prezentat modificări fenotipice comparativ cu plantulele control care nu au fost supuse conservării. Rezultatele obținute au demonstrat că vitroculturile de cartof soiul Mureşan pot fi conservate pentru o perioadă de cel puțin un an, fără subcultură, sub un strat de ulei de parafină cultivate fiind pe medii conținând acid abscisic, cu menținerea a capacității regenerative a acestora după transferul lor în condiții optime de cultură.

Crioconservarea prin vitrificare. Apexurile caulinare de cartof au fost congelate printr-o modificată metodă a vitrificării. Ca urmare a tratamentului cu soluții concentrate de crioprotectori apexurile caulinare au supraviețuit congelării în azot lichid şi au regenerat plantule după decongelare şi transferul lor pe medii de cultură favorabile regenerării de plantule.