Acta Physiol Plant (2015) 37:234
DOI 10.1007/s11738-015-1983-8

ORIGINAL ARTICLE

In vitro slow growth storage of *Senecio macrophyllus* shoots

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Received: 27 March 2015 / Revised: 21 July 2015 / Accepted: 5 October 2015 / Published online: 17 October 2015

Abstract Advances in biotechnology, especially in the field of in vitro culture techniques, led to the development of procedures that can be used as an excellent tool in plant conservation. The present study describes in vitro conservation of *Senecio macrophyllus* by slow-growth storage. Various sugar treatments, concentration of abscisic acid (ABA), light intensity and type of containers were tested. Viability and proliferation rate of shoots were evaluated 4 weeks after regrowth. The results obtained showed that polycarbonate boxes were a better type of containers for storage of *S. macrophyllus* shoots than glass vessels. Light negatively affected culture viability, multiplication rate and rooting response. In the case of *Senecio macrophyllus* the addition of ABA to storage medium stimulated survival and higher proliferation of shoots during regrowth in optimal conditions in comparison to shoots stored on medium without ABA. Shoots of *S. macrophyllus* were effectively stored for 6 months. All rooted shoots survived adaptation to field conditions and were able to flower in the second year after acclimatization.

Keywords In vitro storage · *Senecio macrophyllus* · Shoot tips · ABA

Communicated by M. Lambardi.

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Introduction

The latest International Union for Conservation of Nature statistics show that out of over 12,000 plant species, 70 % are threatened, 19 % are critically endangered and 28 species are extinct in the wild. In situ conservation alone may not be enough to rescue some of the rarest plants (Sarasan 2010). Besides the classical forms of protection of endangered species in the past three decades, advances in biotechnology and especially in the field of in vitro culture techniques led to the development of procedures that can be used as an excellent tool in plant protection (Maryam et al. 2014). The most important aspects of this protection are the development of efficient regeneration systems for many endangered species, that is micropropagation, and the protection of the morphogenetic potential of cell cultures, tissue or organs (Rybczyński and Mikuła 2006). Although standard in vitro propagation methods are available, endangered species may have unusual growth requirements and thus may require modified procedures for in vitro culture (Reed et al. 2011).

In vitro cultures used for the micropropagation of plants are an excellent tool to protect endangered species. Clonal propagation techniques need to create culture conditions conducive to intensive growth. Media used for micropropagation are supplemented with growth regulators and trophic ingredients in appropriate proportions and the cultures are carried out in an optimal growth temperature and light intensity. The cultures run in optimal conditions; however, they require passage to fresh medium every few weeks, and the procedure is laborious, costly and fraught with the risk of somaclonal variation and microorganism contamination. In vitro culture may also be used for conservation of plant genetic resources. The development of procedures for slow growth led to maintaining the cloned

material for a few months to several years (depending on the species) under tissue culture conditions, requiring only infrequent subculturing (Rao 2004). Slow growth of in vitro cultures can be achieved by reducing the osmotic potential of the storage medium, lowering incubation temperature or altering medium composition and photon flux density (Withers and Engelmann 1997). The most important factor in the slow growth culture is temperature reduction, often with a simultaneous decrease in light intensity or even culture in the dark, which limits shoot growth from callus induction and proliferation of the base of the shoots. According to the data this minimizes further growth and the development of plantlets or other explants is already guaranteed by the use of a temperature of 0–10° C (Orlikowska 1992; Hausman et al. 1994; Reed 2002). Furthermore, by modifying the composition of the medium mainly by reducing the sucrose content and/or minerals, the use of hormonal growth retardants, e.g. abscisic acid (ABA) or osmotic agents such as mannitol and sorbitol, inhibition of cell division can be achieved, which significantly limits the growth of both callus and shoot formation (Lambardi et al. 2006; Moges et al. 2003; Shibli et al. 2006). Moreover, the addition of ABA to the medium, especially in cold conditions, increases tolerance to dehydration of tissues and stimulates their survival (Kobayashi et al. 2008). Post-storage vigour of the explants depends on different storage conditions, which include temperature, lighting conditions, medium composition, time of storage, type of container and plant genotype (Hausman et al. 1994; Kovalchuk et al. 2009).

The aim of the study is to determine the conditions for storage of shoots of *Senecio macrophyllus* in slow-growing cultures and the survival of cultures, and their proliferation in the first passage at normal temperature after storage at 10 °C in light or dark, in relation to media used during cold storage. *Senecio macrophyllus* M. Bieb. (Astreaceae) is an extremely rare species and is listed in the *Polish Red Data Book* (Zarzycki and Szeląg 2006). Cultures in vitro used for the micropropagation of *Senecio* could help in its conservation, because its seeds completely lose their ability to sprout after 1 year, making it unfeasible to set up a seed bank.

Materials and methods

Plant material

Senecio macrophyllus shoot tips were micropropagated in 300 ml glass jars with 50 ml MS medium with 4.4 μM benzyladenine (BA), 0.54 μM 1-naphthaleneacetic acid (NAA) and 0.8 % agar. Shoots were cultured in continuous white fluorescent light (80 μmol m^{-2} s^{-1}) at 26 ± 1 °C and subcultured on a 4-week cycle prior to storage (Trejgell et al. 2010).

Storage conditions

In the first experiment, shoot tips isolated from the cluster were stored in 300 ml glass jars and 300 ml polycarbonate magenta boxes with polypropylene cover (both vessels) that contained 50 ml half-strength MS (Murashige and Skoog 1962) medium (1/2MS) supplemented with 2.2 μM BA and 0.27 μM NAA. Three carbohydrate treatments were tested: 3 % sucrose (3 % sc), 1.5 % sucrose plus 1.5 % sorbitol (1.5 % sc + sb), and 3 % sucrose plus 3 % sorbitol (3 % sc + sb). The experiment was conducted in darkness.

In the second experiment, shoot tips were kept in magenta boxes containing ½ MS with different combinations of carbohydrates, supplemented with 2.2 μM BA and 0.27 μM NAA and additionally with ABA at a concentration of 3.8 or 9.5 μM. Shoot tips stored on medium without ABA were the control. The experiment was conducted in darkness or in continuous white fluorescent light (40 μmol m^{-2} s^{-1}).

The pH of all types of medium was adjusted to 5.8 before autoclaving. The media were sterilized for 20 min at 120 °C. All stored cultures were incubated in a growth room with reduced temperature (10 °C) for 3, 6 or 9 months in darkness.

Data collection

The shoots were evaluated at 3-month intervals in accordance with the schema procedure reported in Fig. 1. Analysis of the visual rating was conducted directly after transfer to optimal growth conditions. Shoots were rated on the scale from 0 to 5, based on plant appearance (Reed 1992, modified): 0—dead shoot, all brown, 1—shoot mostly brown, only extreme shoot tip visibly yellow, 2—shoot yellow–brown, 3—shoot with etiolated tip, base of

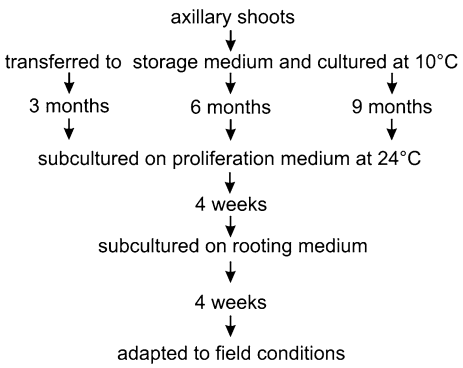

Fig. 1 Schematic procedure of storage and regrowth of *S. macrophyllus*

stem brown, 4—shoot with etiolation, 5—shoot bright green. Viability and proliferation rate of shoots were evaluated 4 weeks after transfer to optimal growth conditions. Shoots were subcultured on MS medium with full-strength nutrients, supplemented with 4.4 μM BA and 0.54 μM NAA under continuous white fluorescent light (80 μmol m^{-2} s^{-1}) at 26 ± 1 °C. The shoots obtained were excised and transferred on rooting medium (MS without auxin). The percentage of rooted shoots and the number of roots per shoot were recorded after 4 weeks of culture. The plantlets were removed from the in vitro cultures, washed gently with sterile water, transferred to plastic pots containing a sterile mixture of vermiculite and sand (1:1 v/v), and covered with transparent boxes to maintain humidity (60–70 %). After 8 weeks of acclimatization the survival rates were determined, and then the plantlets were transferred to field conditions. The survival level and the ability to flower were analysed during 2 years.

Data analysis

In each variant of storage 30 shoots were used (six shoot tips in each magenta/jar). The experiments were conducted twice. Data on regrowth under optimal conditions (multiple rate and percentage of rooted shoots and number of roots per shoot) were obtained for shoots that survived the cold treatment. Data were expressed as mean ± standard error (SE) and analysed by ANOVA. To examine significant differences among the treatments Tukey's multiple range test at $p < 0.05$ was then performed.

Results

The visual rating of shoot tips was 5 on establishment of culture, decreasing with the duration of storage. Analysis of the visual rating in the first experiment showed that for each of the media the value was higher when shoot tips were stored in magenta boxes compared to plant material stored in glass jars (Table 1; Figs. 2, 3a, b). After 9 months of storage in magenta boxes the visual rating ranged from 1.0 to 1.6. The highest value was recorded for the shoots planted on medium with 3 % sucrose (Table 1).

Shoot tips after 3, 6 and 9 months of storage at 10 °C were transferred to optimal growth conditions. Multiple rates recorded after regrowth following the 3 months of storage in magenta boxes on medium with 4.4 BA were lower compared to results obtained during multiplication of non-stored shoots on a medium of the same composition of growth regulators, but similar to results recorded on a medium containing 2.2 BA and 0.27 NAA (Trejgell et al. 2010). By contrast, the multiple rate for shoots stored in glass jars was about half that and differences were statistically significant (Table 1). Extension of the storage to 9 months significantly reduced the multiple rate. The best responses were observed when shoots were stored on medium with 1.5 % sucrose plus 1.5 % sorbitol: multiple rates were 2.3 (Fig. 3c) and 1.0 after 6 and 9 months, respectively (Fig. 4a). ANOVA indicated significant interaction between the type of container and carbohydrate treatment during shoot multiplication after 6 and 9 months of storage (Table 1). Magenta boxes were a better type of container for storage of *S. macrophyllus* shoots.

Table 1 Analysis of the interaction between the type of container and sugar treatment during cold storage on the visual rating and during post-storage multiplication of shoots and rooting of *S. macrophyllus* performed by two-way ANOVA

Treatments	Visual rating			Multiple rate			Rooting rate		
	MS	*F*	*p* value	MS	*F*	*p* value	MS	*F*	*p* value
3 months of storage									
Type of container (A)	19.38	38.72	<0.0001	49.01	31.58	<0.0001	336.40	144.76	<0.0001
Sugar treatment (B)	13.07	26.11	<0.0001	0.90	0.58	0.5625 ns	37.64	16.20	<0.0001
A × B	0.88	1.76	0.1751 ns	0.02	0.01	0.9867 ns	4.13	1.78	0.1751 ns
6 months of storage									
Type of container (A)	38.52	86.07	<0.0001	22.69	17.82	<0.0001	14.40	11.86	0.0009
Sugar treatment (B)	0.38	0.85	0.4293 ns	16.35	12.84	<0.0001	16.58	13.65	<0.0001
A × B	9.72	21.73	<0.0001	9.89	7.77	0.0006	41.20	33.93	<0.0001
9 months of storage									
Type of container (A)	34.17	98.97	<0.0001	22.69	17.82	<0.0001	0.9	4725	0.0325
Sugar treatment (B)	5.36	15.52	<0.0001	16.35	12.84	<0.0001	0.3	1575	0.2131 ns
A × B	0.98	2.85	0.0603 ns	9.89	7.77	0.0006	0.3	1575	0.2131 ns

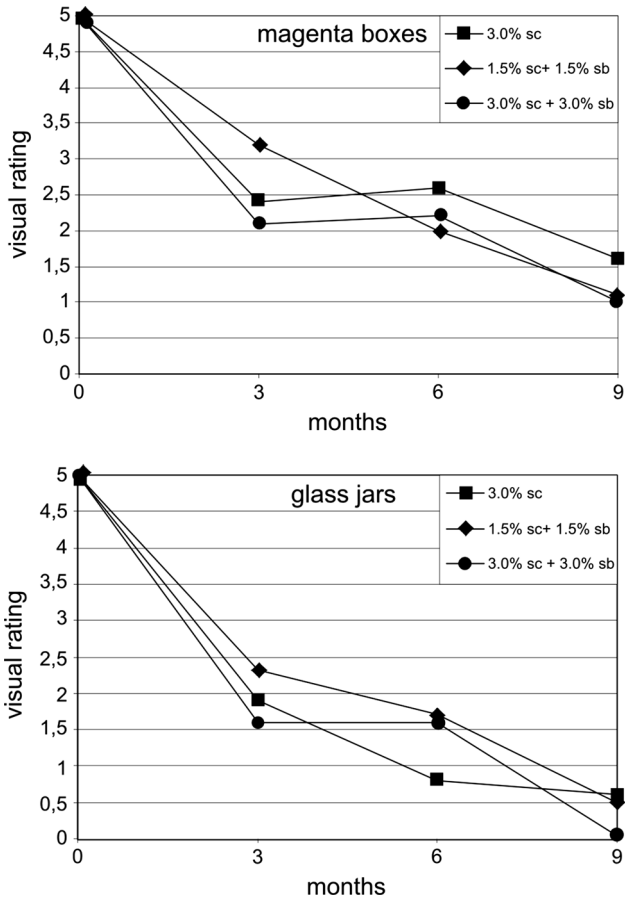

Fig. 2 The effect of carbohydrate treatment during storage in glass jars and magenta boxes on the visual rating of *S. macrophyllus* shoots

The axillary shoots obtained on stored material were rooted. During regrowth following 3 and 6 months of storage on all types of media and in both types of containers, shoots were able to root (Figs. 3d, 4b), but the rooting response of shoots stored for 6 months on all variants of medium and in both types of containers was significantly lower. Root development on the shoots obtained following 9 months of storage was observed only when magenta boxes were used for storage on medium with 1.5 % sc + sb and 3 % sc + sb, but the percentage of rooted shoots did not exceed 20 % and the number of roots per shoot was 0.4 and 0.2, respectively (Fig. 4b, c). The shoots stored on medium with 3 % sucrose in magenta boxes and in glass jars after regrowth were not able to root (Fig. 4b, c). The type of container used for storage and the combination of sugar used in the culture medium had a significant effect on the rooting of shoots, but there was no correlation between these treatments (Table 1).

Rooted shoots (100 %) adapted to ex vitro conditions (Fig. 3e) and all of them were able to flower in the second year after transfer into the field (Fig. 3f).

In the second part of the study the effect of light on the survival of apical growth was investigated. Analysis of the visual rating showed that the light has a negative effect on the storage of *S. macrophyllus* shoots. During the first 3 months of storage the greatest changes were recorded in the morphology of the material stored on the medium with 3 % sucrose and 1.5 % sucrose with sorbitol and the visual rating was not higher than 1, and in the following months of storage it was 0.6–0.3 (Fig. 5). After 3 months of storage on the medium with 3 % sucrose and 3 % sorbitol chlorosis lamina and necrotic changes to leaves on stored shoots were observed and the visual rating was 1.6, which decreased during subsequent storage to a value of 0.5 after 9 months of storage. Storing the shoot in the dark on analogous media did not cause such destructive changes, and the value of the visual rating after 6 months of storage was greater than 2. The addition of ABA to the medium favoured the shoot stored both in the dark and the light. The highest visual rating (2.7) was reported for the shoot stored in the dark on medium with 1.5 % sucrose and 1.5 % sorbitol supplemented with ABA at a concentration of 3.8 and 9.5 μM (Fig. 5). Analysis of the interaction of light conditions and medium on the visual rating after cold storage showed that differences were statistically significant (Table 2).

After transfer to the optimal conditions for the proliferation of shoots, multiple rates were recorded and the highest efficiency of multiplication of shoots (3.7 per explant) was shown by shoots stored for 3 months in darkness on medium with 1.5 % sc + sb with the addition of 3.8 μM ABA (Fig. 6a). In comparison after 6 and 9 months of storage shoots showed significantly higher rates of proliferation for shoots stored on medium with 3 % sc and 1.5 % sc + sb with ABA regardless of the concentrations used and on medium with 1.5 % sc + sb without the addition of ABA. The number of shoots per explant oscillated between 2.2 and 2.6 after 6 months of storage, and after 9 months was in the range 1.0–1.3 (Fig. 6a).

When the shoots were stored in light higher multiple rates for shoots stored for 3 months were recorded on the medium 1.5 % sc + sb and 3 % sc + sb with the addition of ABA, regardless of the concentration used, whereas after 6 months only on medium with 3 % sc + sb and 3.8 μM ABA (Fig. 6a). After the extension of storage to 9 months only a few shoots were viable, and their proliferation was inhibited. Light conditions used for storage and the type of culture medium and correlation between these treatments had a significant effect on the multiplication rate (Table 2).

The shoots obtained were able to root, but the extension of storage time inhibited this process. This was particularly visible when the shoots developed from material stored in light for 6 and 9 months. Only in the case of shoots propagated from the material stored on the medium with 3 % sucrose and 3 % sorbitol and 1.5 % sucrose and 1.5 %

Fig. 3 Storage in vitro of *S. macrophyllus* shoots during 3 months in a glass jar (**a**) and in a magenta box (**b**) on medium with 1.5 % sucrose and 1.5 % sorbitol, **c** regrowth of shoots on medium with BA 1.0 mg/L^{-1} and NAA 0.1 mg/L^{-1} after 6 months of storage in a magenta box, **d** rooted shoot on medium without plant regulators, **e** plantlets adapted to field conditions, **f** flowered plants 2 years after acclimatization

sorbitol without the addition of ABA, light conditions did not inhibit rooting (Fig. 6b, c). The addition of ABA to the medium during storage in most variants led to a reduction in both the percentage of rooted shoots as well as the number of roots on the shoot (Fig. 6b, c). Light conditions and the type the culture medium for a duration of 3 and 6 months of storage, as well as the correlation between these treatments had a significant effect on the rooting rate. In contrast, after 9 months of storage significant differences in the rooting rate were observed only on shoots stored in different light conditions (Table 2). Developed roots were short and fairly brittle. All rooted shoots acclimatized to ex vitro conditions, 100 % of plantlets survived transfer to field conditions. Plants developed normally and were able to flower.

Discussion

Long-term in vitro storage has been successfully improved by applying different treatments, of which reduction of temperature seems to be one of the most important. Plant growth during storage can be suppressed by lowering the temperature, but the optimal storage-temperature depends on species. Typically, a range of 1–10 °C will be appropriate for temperate species. However, many species growing well in this climate zone are chill-sensitive and somewhat higher temperatures of 6–12 °C are used for them. Better results of storage were achieved at a temperature of 8 °C for *Castanea sativa* (Capuana and Di Lonardo 2013), but for *Beta vulgaris*, *Solanum* sp and *Vitis rupesttris* a temperature of 9–12 °C was used (Grout 1995). During storage of the shoot tips of *S. macrophyllus* a temperature of 10 °C was applied. This temperature was also the best during storage of the axillary buds of *Hosta tokudama* (Gollagunta et al. 2005), *Podophyllum peltatum* (Lata et al. 2010) and *Saintpaulia ionantha* (Moges et al. 2003).

Different types of containers can have an impact on the condition and survival of plant material stored in vitro. In our study, we tested two types of containers, magenta boxes and glass jars. The results obtained showed that the polycarbonate boxes were a better type of container for storage of *S. macrophyllus* shoots than glass vessels. In our study we used vessels with covers of the same material, therefore the differences in storage could be affected by the material from which vessels were made. Glass conducts heat better than polycarbonate, so perhaps plant material in the jars was more chilled than the material stored in Magenta boxes. Polycarbonate magenta boxes were also successfully used for cold storage of *Solanum tuberosum, Prunus virginiana* (Pruski et al. 2000) and for *Pistacia*

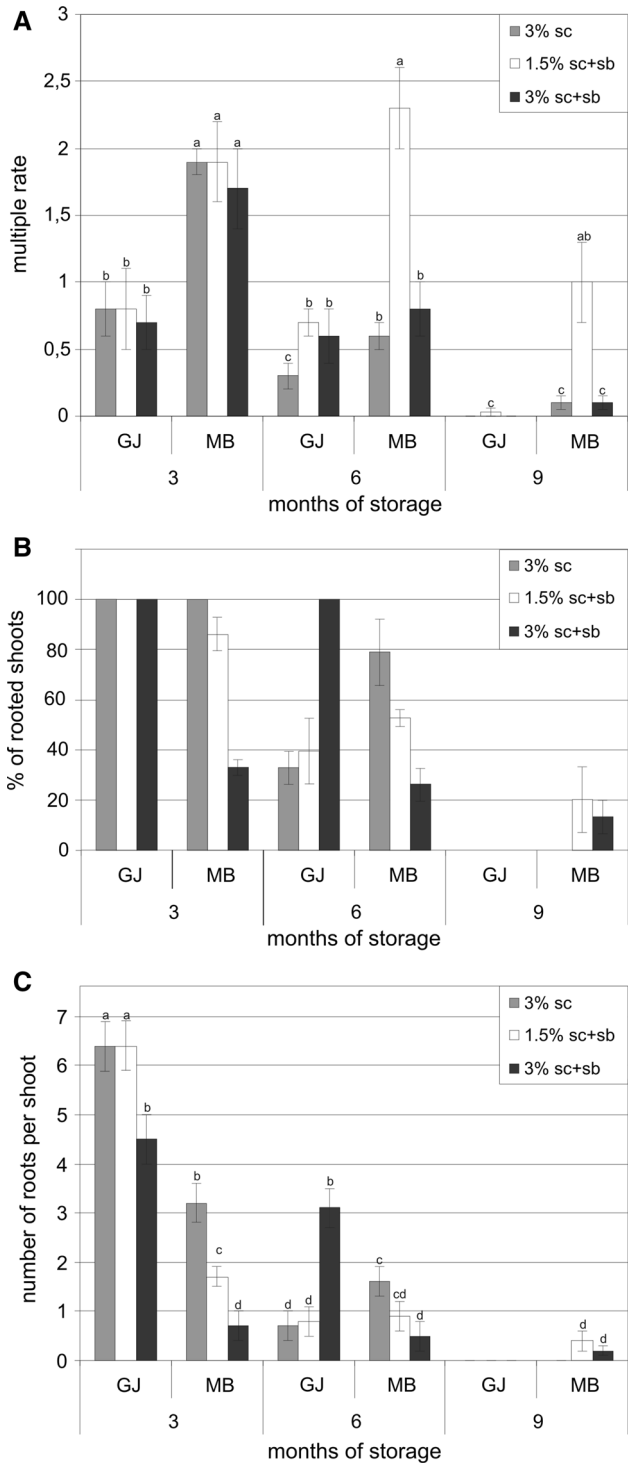

Fig. 4 The effect of carbohydrate treatment and the type of container on multiple shoots (**a**), % rooting of shoots (**b**) and number of roots per shoot (**c**) of *S. macrophyllus* after storage at 10 °C (*GJ* glass jars; *MB* magenta boxes)

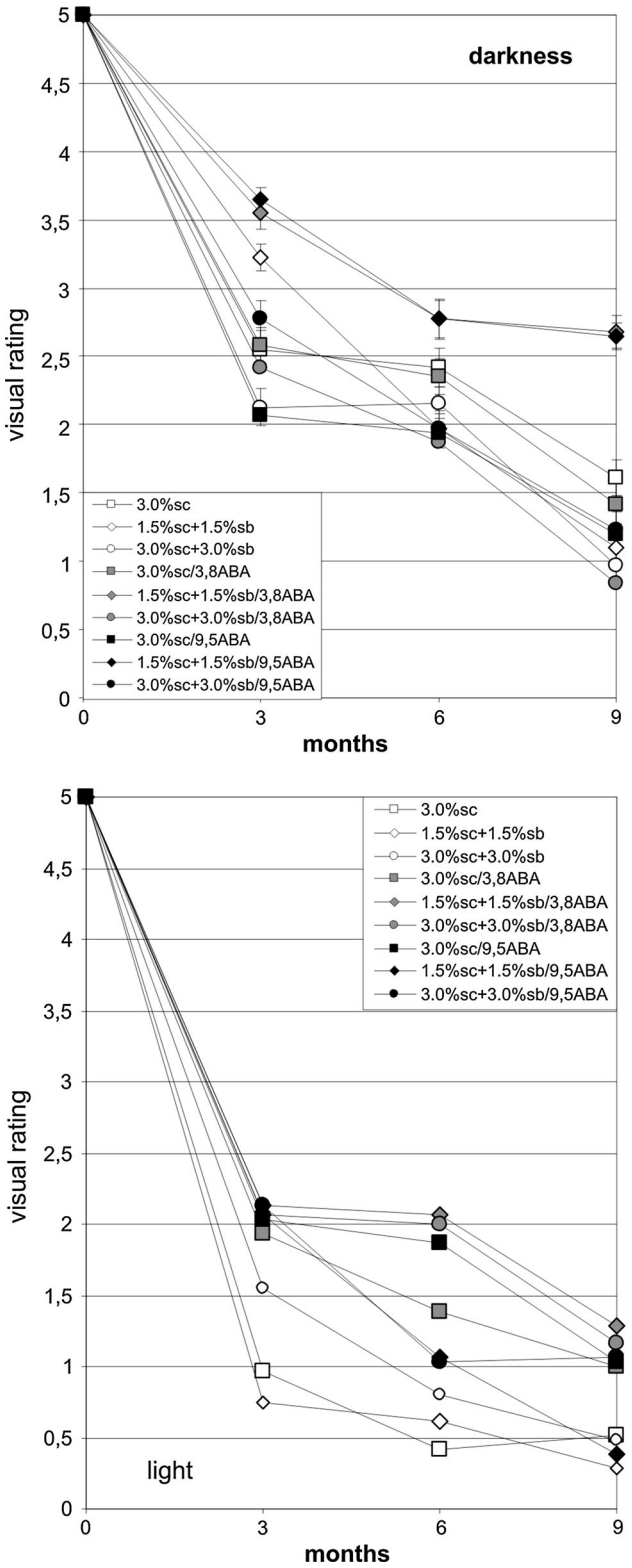

Fig. 5 The effect of carbohydrate treatment during storage in darkness and light conditions on the visual rating of *S. macrophyllus* shoots

lentiscus (Koç et al. 2014). Similar results were obtained by Kovalchuk et al. (2009) for two varieties of apple, where shoots stored in semi-permeable plastic bags

Table 2 Analysis of the interaction of light conditions and medium during cold storage on the visual rating and during post-storage multiplication of shoots and rooting of *S. macrophyllus* performed by two-way ANOVA

Source of variation	Visual rating			Multiplication rate			Rooting rate		
	MS	*F*	*p* value	MS	*F*	*p* value	MS	*F*	*p* value
3 months of storage									
Light conditions (A)	154.903	262.723	<0.0001	67.377	76.091	<0.0001	23.115	15.961	<0.0001
Medium (B)	10.187	17.278	<0.0001	17.429	19.683	<0.0001	17.320	11.960	<0.0001
A × B	8.758	14.854	<0.0001	7.674	8.666	<0.0001	23.465	16.203	<0.0001
6 months of storage									
Light conditions (A)	133.565	250.658	<0.0001	53.777	37.519	<0.0001	8.533	9.339	0.0025
Medium (B)	8.012	15.036	<0.0001	15.035	10.489	<0.0001	8.548	9.355	<0.0001
A × B	8.367	15.702	<0.0001	10.266	7.162	<0.0001	8.567	9.375	<0.0001
9 months of storage									
Light conditions (A)	71.685	179.587	<0.0001	70.840	214.249	<0.0001	2.7	15.997	<0.0001
Medium (B)	9.698	24.295	<0.0001	3.828	11.578	<0.0001	0.192	1.135	0.3397 ns
A × B	9.334	23.383	<0.0001	3.434	10.386	<0.0001	0.192	1.135	0.3397 ns

remained in good condition for 9 months; however, the use of glass vessels allowed storage to be extended for up to 18 months.

Medium composition also influences plant survival and growth during storage. In our study we used half-strength MS medium and standard amounts of sucrose, with or without sorbitol at the same concentration (3 %) or sucrose reduced to half of the quantity with the addition of sorbitol (1.5 %), supplemented with 2.2 µM BA and 0.27 µM NAA. Reducing the concentration of MS nutrients gave good results during the storage of *Lilium longiflorum* and *L. henryi* (Bonnier and Van Tuyl 1997). In contrast, during storage of *S. macrophyllus* shoots, reducing the concentration of MS nutrients, growth regulators and the addition of sorbitol to the medium did not limit the necrotic lesions and slightly inhibited shoot growth during storage. A negative correlation was found between strong growth during storage and viability and regrowth after storage, because a significant reduction in regrowth was observed following storage, in comparison to shoots propagated without cold storage where the rate was more than 8 shoots per explant (Trejgell et al. 2010). However, after 6 and 9 months of storage the replacement of 3 % sucrose in the medium on a mixture of 1.5 % sucrose and 1.5 % sorbitol resulted in the highest rate of multiplication among all treatments. Lower rates of proliferation after storage of shoots on medium with a reduced content of sucrose may be due to reduced carbohydrate reserves in the tissues and may lead to a reduction in the potential for regeneration. The addition of mannitol to the medium also improved the survival of the microplant *Solanum tuberosum* (Sarkar and Naik 1998) and several cultivars of *Malus domestica* (Kovalchuk et al. 2009). However, the addition of mannitol to the storage medium negatively affected the survival and multiplication capacity of microcuttings of *Cedrus atlanticus* and *C. libani* (Renau-Morata et al. 2006).

The presence of cytokinins in the medium could affect the stimulation of the cellular metabolism (cell division); on the other hand, during storage of the shoots, there was no root development. The presence of cytokinins is associated with the appearance of necrosis on explants during cold storage, but the lack of roots leads to a decrease in endogenous cytokinin biosynthesis and a consequent reduction in cell division (Bairu et al. 2009).

The influence of light intensity during storage has been studied for different species. The best results have been observed when storage was performed in darkness for *Malus* (Orlikowska 1992), *Pistacia* (Koç et al. 2014), *Populus* (Hausman et al. 1994), and *Quercus* (Romano and Martins-Loução 1999). Similar results were reported for *S. macrophyllus* where light affected negatively culture viability, multiplication rate and rooting response. Storage in low levels of illumination (2–20 µmol m^{-2} s^{-1}) was reported for *Fragaria* (Reed 2002), *Lilium* (Bonnier and Van Tuyl 1997), *Hosta* (Gollagunta et al. 2005), and *Solanum* (Sarkar and Naik 1998). However, shoots of *Castanea sativa* were stored under the conditions of only 30 % reduced illumination (Capuana and Di Lonardo 2013), but shoots of *Podophyllum peltatum* in a light level of approximately 52 µmol m^{-2} s^{-1} (Lata et al. 2010).

ABA is known as a growth retardant on in vitro growth. ABA treatment usually increases cold tolerance in stored plant material by increasing the expression of COR/LEA genes (Kobayashi et al. 2008) and accumulation of proline (Dörffling et al. 2009). A benefit for shoot survival is also displayed by ABA or/and proline pretreatment of *Brassica campestris* and *B. napus* (Wilen et al. 1994), and *Begonia x erythrophylla* (Burritt 2008). However, pretreatment of

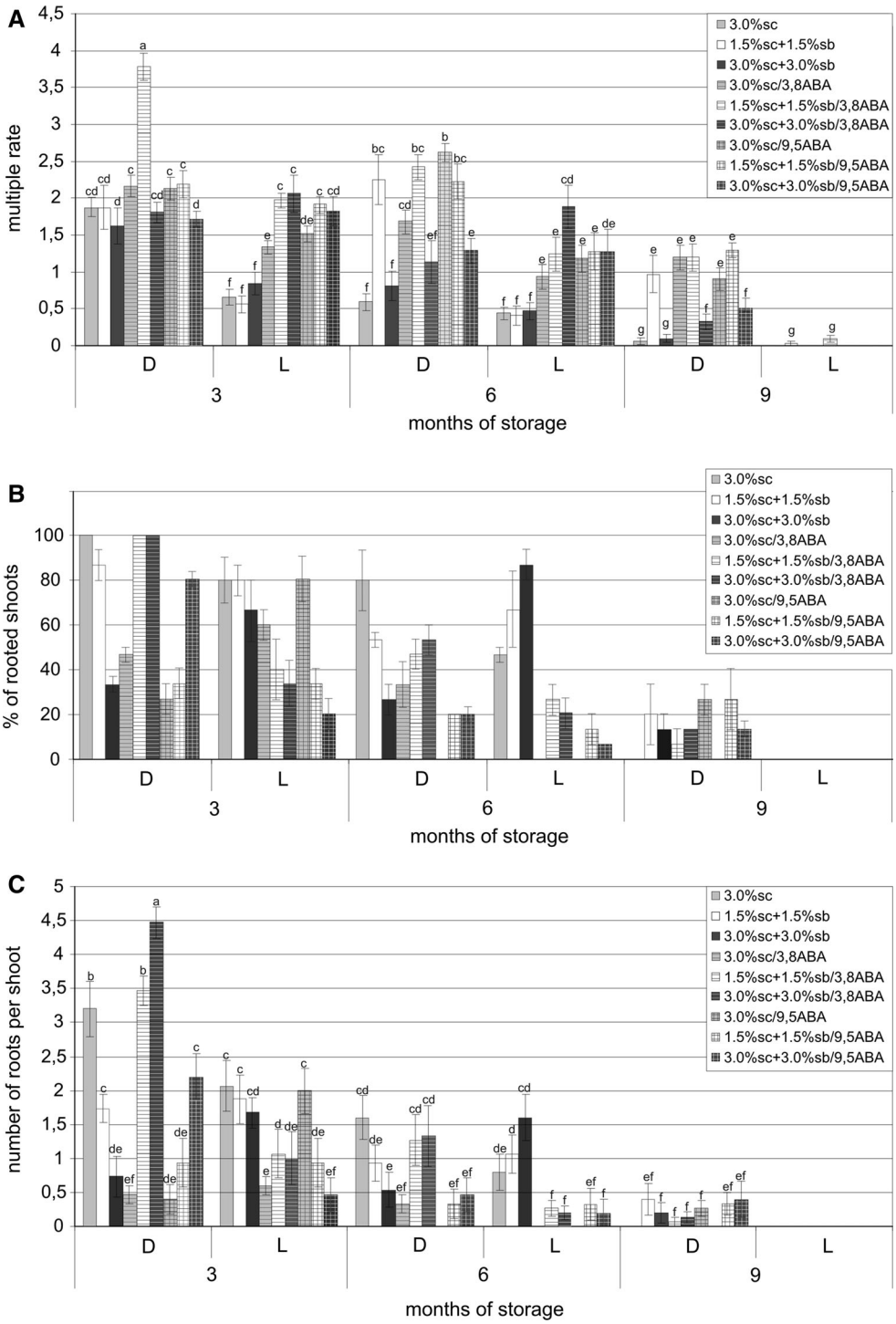

Fig. 6 The effect of carbohydrate treatment and light conditions (*D* darkness; *L* light) on multiple shoots (**a**), % rooting of shoots (**b**) and number of roots per shoot (**c**) of *S. macrophyllus* after storage at 10 °C

ABA did not influence the survival and multiple rate of shoots of *Castanea sativa* (Capuana and Di Lonardo 2013). In the case of *S. macrophyllus* the addition of ABA to storage medium stimulated survival and higher proliferation of shoots during regrowth in optimal conditions in comparison to shoots stored on a medium without ABA. However, the level of multiple rates was lower than for not-stored shoots, probably a long exposure to ABA strongly inhibited the effect of cytokinins.

To summarize, the results obtained demonstrated that *S. macrophyllus* shoot cultures can be stored effectively for up to 6 months without subcultures under a low temperature of 10 °C, in darkness and using magenta boxes. The present study should be a step towards developing an

alternative method for conserving *S. macrophyllus* genetic resources.

Author contribution statement Alina Trejgell designed and carried out the experiments, analysed the data and wrote the manuscript. Monika Kamińska carried out the experiments and helped in writing the manuscript. Andrzej Tretyn helped in preparing the manuscript.

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