

Cryopreservation of hairy root cultures of *Maesa lanceolata* and *Medicago truncatula*

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Received: 27 October 2008 / Accepted: 28 November 2008 / Published online: 11 December 2008
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Abstract To study the production of secondary metabolites of *Maesa lanceolata* and *Medicago truncatula*, hairy root cultures of both plant species were established. Because maintenance of large numbers of cultures is laborious and costly, we developed a cryopreservation protocol and stored different isolated lines over time. Using encapsulation-dehydration, high survival rates were observed for both *Maesa* and *Medicago* hairy roots. Root tips were isolated and encapsulated in calcium-alginate beads, containing 0.1 M sucrose. The encapsulated hairy roots were precultured for 3 days using basal medium containing high sucrose concentrations. *Medicago* root tip growth during the preculturing time lead to unwanted outgrowth which could be tempered by addition of plant growth inhibitors. After preculturing, the beads were dehydrated in the air flow of a laminar flow until 35–40% of the initial bead weight was reached. Dehydrated beads were plunged into liquid nitrogen and after different storage times thawed in a water bath at 40°C. The survival

rates were 90% for *Maesa* and 53% for *Medicago*, which are sufficient to allow implementation in large storage experimental set-ups.

Keywords *Maesa lanceolata* · *Medicago truncatula* · Hairy roots · Cryopreservation · Vitrification · Encapsulation-dehydration

Introduction

Saponins are glycosides of a steroidal or triterpenoid polycyclic structure. They are produced by plants as a defence mechanism against fungal and insect attack (Papadopoulou et al. 1999; Francis et al. 2002). Several types of saponins have been associated with medicinal activity (Sparg et al. 2004). For further investigations on the production of triterpenoid saponins two unrelated plant species were selected.

Maesa lanceolata (*Maesaceae*), also known as False assegai, is a medicinal plant, growing in the tropics of Africa and Asia. The plant is used in traditional medicine in many African countries (Sindambiwe et al. 1996, 1998; Maes et al. 2004; Bagalwa and Chifundera 2007). Bioassay guided fractionation of the methanol extract of dried leaves resulted in the isolation of a saponin mixture (Sindambiwe et al. 1996). The other species investigated is *Medicago truncatula* (*Leguminosae*) or Barrel clover, which produces insecticidal and antinutritional saponins with unfavourable effects on animal feed. *M. truncatula* has emerged recently as a model legume for the study of plant functional genomics and represents also an interesting model species for study and modulation of saponin biosynthesis (Kapusta et al. 2005; Urbanczyk-Wochniak and Sumner 2007).

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To genetically manipulate and evaluate the production of saponins in *M. lanceolata* and *M. truncatula*, hairy roots were established by infecting leaf discs and seedlings with *Agrobacterium rhizogenes*. Hairy roots are more complex structures compared to cell suspensions, and the presence of differentiated cells and tissue has been proposed to ascertain a higher genetic stability (Hu and Du 2006). Other advantages of hairy roots include rapid biomass accumulation, typically accompanied with a high production of secondary metabolites and the possibility for upscaling in specialized bioreactors (Kim et al. 2002; Georgiev et al. 2007).

The down side of the use of in vitro hairy root cultures is that the maintenance is labour intensive and involves culture handling with a high risk of microbial contamination and subsequent loss of original cultures (Grout 1995). Also, prolonged subculturing may affect quantitative or qualitative aspects of secondary metabolite production due to the accumulation of loss of function mutations. The conservation of original cultures using cryopreservation technology can avoid these problems. Storing samples in liquid nitrogen eliminates the need for periodic subculturing and reduces the risk of accumulation of somaclonal variation (Teoh et al. 1996).

Three main techniques can roughly be distinguished for plant cryopreservation; vitrification, encapsulation-dehydration and controlled rate freezing. Despite the progress that has been made in the last decade concerning cryopreservation of plant material, there are only few reports on cryopreservation of hairy root cultures. Vitrification protocols are described for hairy roots of *Atropa belladonna* (Touno et al. 2006), *Panax ginseng* (Yoshimatsu et al. 1996) and *Angelica acutiloba* (Yoshimatsu 2000), while the encapsulation-dehydration method was used for *Vinca minor* (Hirata et al. 2002) and *A Armoracia rusticana* (horseradish) hairy roots (Hirata et al. 1998). One single report describes a slow freezing method for *Artemisia annua* hairy roots (Teoh et al. 1996).

The present research was carried out to establish a robust method for the cryopreservation of hairy roots from saponin producing plants. Vitrification and encapsulation-dehydration procedures were tested and for the latter technique, preculture conditions and dehydration times were optimized.

Materials and methods

Plant material

Maesa lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). The seeds were rinsed in 70% (v/v)

ethanol for 30 s and subsequently surface sterilized with a 70% (v/v) solution of a commercial disinfection product (Haz-tabs; Guest Medical, Kent, UK). After three washes with distilled water, the seeds were placed on Murashige-Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 0.8% (w/v) agar (Lab M plant tissue culture agar MC29, Amersham). Seeds were germinated in a 16/8 h light/dark photoperiod at 26°C.

Medicago truncatula plant material of the cultivar Jemalong J5 was obtained from the group of Marcelle Holsters (VIB—department of Plant Systems Biology—Research group on plant microbes). Seeds of the plants were harvested and scarred with concentrated sulphuric acid. Sterilization was performed using 12% (w/v) sodium hypochlorite. After a 3–4 h treatment with 1 mM 6-benzylaminopurine (BAP), germination of the seeds took place on wet and aseptic Whatmann paper in the dark at 25°C.

Production and maintenance of hairy root cultures

Maesa lanceolata and *M. truncatula* hairy roots were induced using *Agrobacterium rhizogenes* (strain LBA 9402/12) transformation on leaf discs or seedlings. The *Agrobacterium* strain was transformed with the pK7WG2D plasmid, in which an eGFP-ER gene was inserted after a 35S promoter sequence. For both species, the bacteria were grown in the same way. *Agrobacteria* were transferred from a glycerol stock to 3 ml of liquid YEB medium. This medium contained antibiotics (100 mg/l rifampicin, 100 mg/l spectinomycin and 300 mg/l streptomycin) and consisted of 5 g/l beef extract, 1 g/l yeast extract, 5 g/l peptone, 0.15 M sucrose and 2 ml/l 1 M MgSO₂. The bacterial cultures were incubated on a rotary shaker (220 rpm) at 28°C. After 48 h 20 µl of this bacterial preculture was brought into 5 ml of fresh YEB medium with the same antibiotic concentrations as mentioned before. The bacterial cultures were again incubated on a rotary shaker (220 rpm) at 28°C. Forty-eight hours later, these cultures can be used for the transformation. The method for transformation of *Maesa* and *Medicago* was somewhat different. Both procedures are described.

Maesa lanceolata hairy roots were induced by wounding leaf material. Wounded leaves were dipped into the bacterial culture and afterwards placed on solid Murashige-Skoog (MS) basal medium (Murashige and Skoog 1962) supplemented with 0.8% (w/v) agar, 0.06 M sucrose and 1 g/l BA. Because there were no antibiotics in the culture medium, the bacteria could still grow, which led to a co-culture of the bacteria and the leaves. After 3 days of co-cultivation with the *Agrobacteria*, the leaves were placed on solid MS basal medium supplemented with 0.8% (w/v) agar, 0.06 M sucrose and 500 mg/l cefotaxime (Duchefa, The Netherlands) to arrest *Agrobacteria* growth.

Hairy roots were isolated from the leaf material after 15–30 days and were placed on solid Schenk & Hildebrandt (SH) basal medium (Schenk and Hildebrandt 1972) supplemented with 0.8% (w/v) agar and 0.09 M sucrose.

Medicago truncatula hairy roots were induced by wounding seedlings. Wounded seedlings were dipped into the bacterial culture and afterwards placed on solid MS basal medium supplemented with 0.8% (w/v) agar, 0.03 M sucrose and 1 g/l BA. After 7–10 days of co-cultivation with the *Agrobacteria*, the leaves were placed on solid MS basal medium supplemented with 0.8% (w/v) agar, 0.03 M sucrose and 500 mg/l cefotaxime. Hairy roots were isolated from the seedlings after 15–30 days and were placed on MS basal medium supplemented with 0.8% (w/v) agar and 0.03 M sucrose.

Both hairy root cultures were cultured in the dark at 25°C and were subcultured every month. For the selection of the transgenic material, GFP was used as a visible marker. Hairy roots that showed green fluorescence were considered as transgenic.

Vitrification

To test the toxicity of the plant vitrification solution 2 (PVS2: 0.4 M glycerol, 15% (v/v) DMSO, 15% (v/v) ethylene glycol and 0.4 M sucrose) (Sakai et al. 1990), *M. lanceolata* and *M. truncatula* hairy root tips (± 5 mm in length) were excised from 2-week-old root cultures and precultured for 3 days at 25°C in the dark on SH solid medium (*Maesa*) or MS solid medium (*Medicago*) containing 0.3 M sucrose. After the preculture, the samples were transferred to cryovials (10 root tips/cryotube) and incubated at 0°C in 2 ml loading solution (2 M glycerol and 0.4 M sucrose) for 10 min. The loading solution was removed and 2 ml cooled PVS2 solution (0°C) was added to the root tips. Different incubation times (0, 2, 5, 10, 15 and 20 min) with PVS2 solution were tested. After PVS2 incubation, samples were plunged into liquid nitrogen. After storage for 3 days, samples were thawed rapidly in a water bath at 40°C and washed for 5 min with 2 ml of basal medium containing 1 M sucrose. Afterwards the hairy roots were placed on SH or MS solid medium with 0.09 M sucrose.

To assess the toxicity of PVS2, root tips were treated the same way as described above but after treatment with PVS2 solution, the root tips were directly washed for 5 min with basal medium containing 1 M sucrose and were placed on SH or MS solid medium with 0.09 M sucrose. To determine the effect of loading solution on the survival of hairy roots after PVS2 treatment, half of the samples were pretreated with loading solution for 10 min and the other half of the samples were directly incubated in PVS2 solution. Different incubation times with PVS2 were tested (0, 2, 5, 10, 15 and 20 min).

Trypan blue staining was performed for some samples to get an idea about the viability of the hairy roots directly after thawing. For this staining, root tips were put in a drop of trypan blue (Fluka) on a microscopic slide.

However, in all cases the survival rate was recorded as the percentage of hairy roots showing definite elongation or side root formation after cultivation for 6 weeks in the dark at 25°C.

Encapsulation-dehydration

Root tips (2–3 mm in length) were excised from 2-week-old root cultures of *M. lanceolata* and *M. truncatula*. The isolated root tips were immediately suspended in 100 ml of 3% (w/v) sodium alginate solution containing 0.1 M sucrose and dropped into 100 ml 0.1 M CaCl₂, plus 0.1 M sucrose. The roots were kept in the solution for 10 min to allow solidification of the calcium alginate beads. Next, the beads were transferred to a shake flask containing 25 ml liquid SH medium for *Maesa*, or MS medium for *Medicago*, supplemented with 0.5 M sucrose (50 beads/shake flask). After 24 h, the medium was replaced with medium containing 0.75 M sucrose and 24 h later with medium containing 1 M sucrose. Continued growth of *M. truncatula* caused extrusion of the root tips from the alginate beads that render them unsuitable for freezing. Outgrowth of *M. truncatula* root tips was reduced by adding 3 mM ABA (abscisic acid) or 0.01 mM paclobutrazol (Bonzi®) to the preculture media. Optimal concentrations of ABA and paclobutrazol for growth reduction of *M. truncatula* hairy roots were determined in previous experiments (unpublished results).

After 3 days of preculture in medium with increasing sucrose concentrations, the encapsulated root tips were transferred to a Petridish and dehydrated by placing the dish in the air current of a laminar flow hood for 0–5 h, until the bead weight attained ± 35 –40% of the initial weight. Dehydrated beads were placed in cryovials (30 beads/cryovials) and then directly immersed in liquid nitrogen.

After storage for 3 days, the beads were thawed rapidly in a water bath at 40°C and then transferred without washing onto solid SH medium containing 0.09 M sucrose (*Maesa*) or onto solid MS medium containing 0.03 M sucrose (*Medicago*). The survival rate was recorded as the percentage of hairy roots growing out of the beads after cultivation for 6 weeks in the dark at 25°C.

Statistical analysis

Experiments were done in triplicate, each replicate containing a minimum of ten samples. The data represent mean values from the three independent replicates. Data were nonnormal, hence, ranking was calculated using proc

rank (SAS 9_1_3 software). Statistical differences between samples were determined by non parametric oneway analysis of variance, also using SAS 9_1_3 software. Graphs were established using SigmaPlot 10 software.

Results

Induction of hairy root cultures of *M. lanceolata* and *M. truncatula*

Maesa lanceolata and *Medicago truncatula* hairy roots appeared from wounded sites 15–30 days after inoculation with *Agrobacterium rhizogenes* strain LBA 9402/12. Uninfected control explants did not form adventitious roots. GFP was used as a visible marker to select transformed hairy roots. Isolated hairy roots, in contrast to untransformed roots, grew autonomously in hormone free medium. Figure 1a–d represent the hairy root cultures of *Maesa* and *Medicago*, grown in the dark at 25°C on solid and in liquid medium. Hairy roots of *Medicago* grew much faster compared to *Maesa* hairy roots. *Medicago* hairy roots also appeared more fragile, thinner and more yellow than those of *Maesa*. In general, both hairy root cultures grew well on solid and in liquid medium. For the cryopreservation experiments we used hairy roots growing on plates since these were easier to handle.

Cytotoxicity of vitrification solution

One of the major disadvantages of the vitrification protocols using PVS2 is its cytotoxicity (Volk et al. 2006; Xue et al. 2008). PVS2 toxicity tests with *M. lanceolata* and *M. truncatula* hairy roots were preformed prior to freezing. Treatment of *M. lanceolata* root tips with PVS2 solution for more than 2 min strongly reduced their survival (Fig. 2a). After 5 min of PVS2 treatment the survival rate was reduced to 16%. Pretreatment of the roots with loading solution (LS) had a clear positive effect on viability of the *Maesa* hairy roots, maintaining the survival rate at 66% after 5 min of PVS2 treatment. Longer incubation times lead to a strong decrease in viability for both root tips treated with loading solution and without loading solution (Fig. 2a).

Medicago hairy roots also proved to be very sensitive to PVS2 treatment. However, as for *Maesa* hairy roots, pretreatment with loading solution had a positive effect. When PVS2 was directly applied to the *Medicago* hairy roots, survival decreased significantly after 2 min. When the roots were pretreated with loading solution, survival was still 53%. However, survival decreased to 0% after 15 min of incubation in PVS2 solution (Fig. 2b).

Microscopic inspection of root tips, using trypan blue staining, showed that after 15 min incubation time there

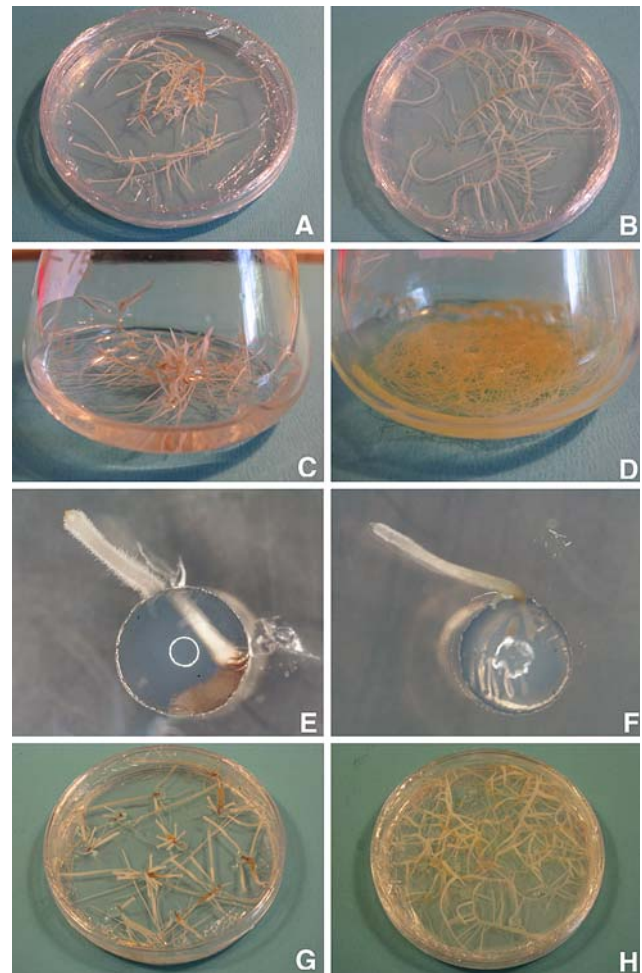


Fig. 1 Morphological features of *M. lanceolata* and *M. truncatula* hairy roots before and after freezing. **a–b** Hairy roots of *Maesa* and *Medicago* on solid medium at 25°C in the dark. **c–d** Hairy roots of *Maesa* and *Medicago* on liquid medium at 25°C in the dark, 120 rpm. **e–f** Hairy roots of *Maesa* and *Medicago* growing out of the calcium-alginate beads, 2 weeks after thawing. **g–h** Hairy roots of *Maesa* and *Medicago* after cryopreservation, allowed to recover for 4 weeks

were no more living cells in the *Medicago* and *Maesa* root tips. The high toxicity of the PVS2 solution to *Maesa* and *Medicago* root tips, disallowed recovery of the material after liquid nitrogen freezing was performed. For the freezing experiments, different PVS2 incubation times were tested (0, 2, 5, 10, 15 and 20 min) and part of the samples was treated with loading solution prior to incubation in PVS2 solution. Neither of these conditions led to regeneration of *Maesa* or *Medicago* hairy roots after freezing in liquid nitrogen (results not shown).

Encapsulation-dehydration of hairy roots from *M. lanceolata* and *M. truncatula*

The application of the encapsulation-dehydration protocol is considered to be more complicated than vitrification and

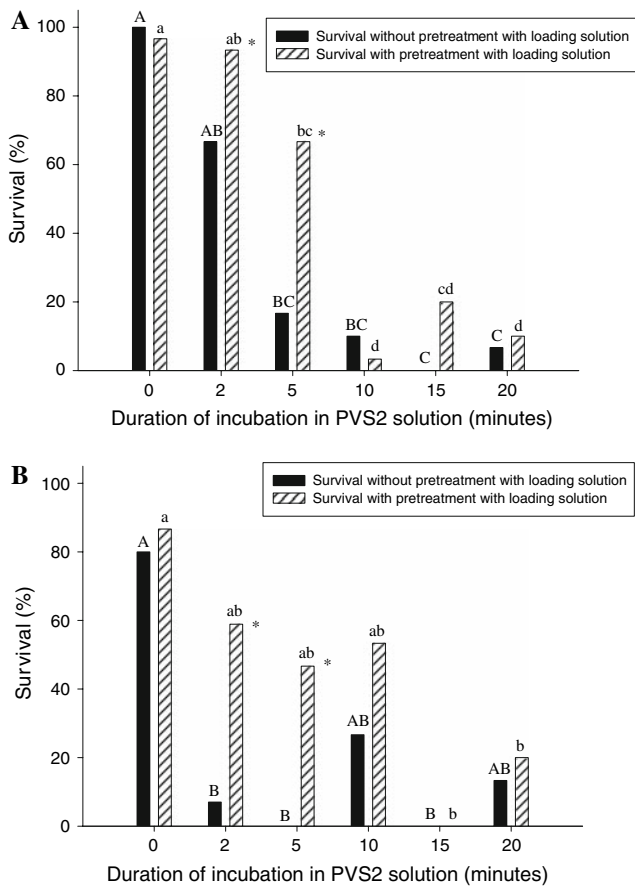


Fig. 2 The effect of PVS2 treatment on hairy roots of (histogram a) *M. lanceolata* and (histogram b) *M. truncatula*, prior to freezing. Different incubation times in PVS2 solution were tested. Also the influence of 10 min pretreatment with loading solution was investigated for both *Maesa* and *Medicago*. Each experiment consisted of three repeats with at least 10 hairy roots in each repeat. Different letters indicate significant differences ($P < 0.05$), “*” significant difference ($P < 0.05$) between two different treatments on the same time point; according to non parametric ANOVA

requires additional testing before it can be implemented for a many species.

Dehydration to very low intracellular moisture levels, so that crystallisation cannot occur, is essential for the survival of cryopreserved hairy roots. An experiment was therefore set up to determine the maximal dehydration time for encapsulated *Maesa* and *Medicago* hairy roots. Hairy roots were dehydrated for up to 5 h. Concurrently, they were weighed every half hour and the percentage of the initial bead weight was determined. The weight loss of the beads was similar for *Maesa* and *Medicago* hairy root beads (Fig. 3a). The weight of the beads decreased rapidly until 2 h of dehydration. From then on the weight of the beads was approximately constant, reaching a minimum after 5 h of dehydration when 35% of the initial bead weight was achieved for *Maesa* and 36% for *Medicago*.

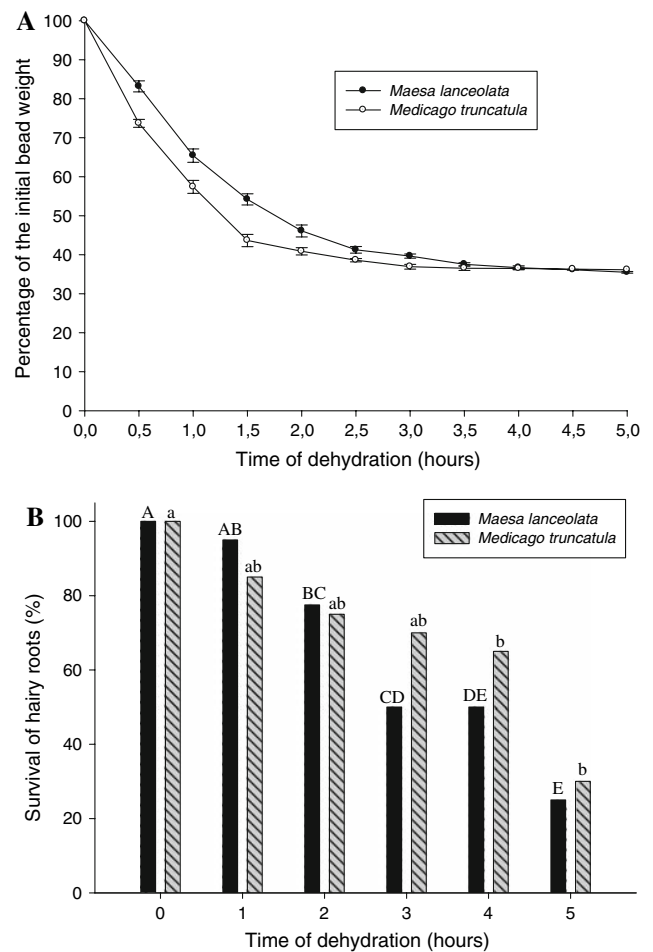


Fig. 3 Control experiments to determine the optimal dehydration time for *M. lanceolata* and *M. truncatula* (without freezing). (Curve a) Encapsulated hairy roots of *Maesa* and *Medicago* were dehydrated for 5 h by the air of the laminar flow cabinet. Beads were weighed every 30 min. (Histogram b) Survival of the dehydrated hairy roots of *Maesa* and *Medicago*. Beads were put on a plate with solid medium and survival rates were recorded 6 weeks after thawing. Each experiment consisted of three repeats with at least 10 hairy roots in each repeat. Different letters indicate significant differences ($P < 0.05$), “*” significant difference ($P < 0.05$) between two different treatments on the same time point; according to non parametric ANOVA

Every hour during dehydration, control beads were placed on solid medium to regenerate. After 6 weeks the survival of the hairy roots was evaluated (Fig. 3b). As expected, the survival of the dehydrated controls decreased with increasing dehydration time for both *Maesa* and *Medicago*. There were no significant different effects for *Maesa* and *Medicago*. The survival before dehydration was 100% for both species (Fig. 3b, time point 0) and was 25 and 30% after 5 h of dehydration for *Maesa* and *Medicago*, respectively. For both *Maesa* and *Medicago*, there is a continuous decline in survival percentages with no significant drops (Fig. 3b).

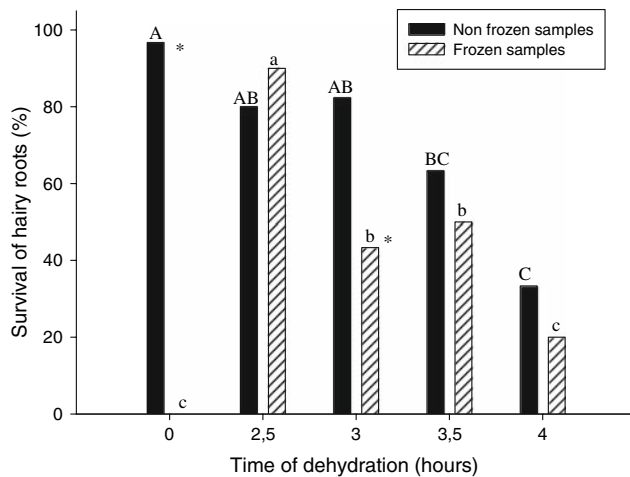


Fig. 4 Survival rates of *M. lanceolata* hairy roots after cryopreservation. Hairy root beads were dehydrated for different time points before freezing. Survival rates were recorded 6 weeks after thawing. Each experiment consisted of three repeats with at least 10 hairy roots in each repeat. Different letters indicate significant differences ($P < 0.05$), ‘*’ significant difference ($P < 0.05$) between two different treatments on the same time point; according to non parametric ANOVA

Freezing of 0–4 h dehydrated *M. lanceolata* hairy root tips was tolerated (Fig. 4). Survival of 90% of the hairy roots was observed after 2.5 h of dehydration, which represents no significant difference to the unfrozen controls. After 4 h of dehydration, survival rate dropped to 20% after freezing. Because of these positive results, no further optimization of the protocol was attempted for *M. lanceolata* hairy root conservation.

The survival after encapsulation-dehydration of *M. truncatula* was much lower compared to *M. lanceolata* (Table 1). The highest survival rate (16%) was obtained with samples dehydrated for 5 h. However, during the 3 days of preculture, already 24% of the hairy root tips had grown out of the bead. This meant that about 1/4 of all hairy roots were not protected anymore against freezing damage. To prevent this outgrowth, we included the growth inhibitors abscisic acid (ABA) and paclobutrazol. These substances were added solely to the preculture media and not to the alginate or calcium solutions, to avoid negative effects after thawing. Using ABA, still 19% of the hairy roots had grown out of the bead after the preculture. Addition of paclobutrazol lowered this percentage to 7%. The survival rates of the hairy roots after freezing are given Table 1. The highest survival rate using ABA as a growth inhibitor was 30%, while the highest survival rate using paclobutrazol as a growth inhibitor was 55%. Both ABA and paclobutrazol clearly inhibit growth of the hairy roots during preculture and don’t have a negative effect on the viability of the hairy roots which results in higher survival

Table 1 Comparison of the effect of different growth inhibitors during preculture, on the survival of *M. truncatula* hairy root tips

Growth inhibitor	Freezing	Dehydration time	Survival (% \pm SE)	
None	No	0	92.7 \pm 3.7 a	
	No	2	60.0 \pm 15.3 ab	
	No	3	60.0 \pm 10.0 ab	
	No	4	26.7 \pm 3.3 b	
	No	5	34.8 \pm 10.6 b	
	Yes	0	0.0 a	
	Yes	2	3.3 \pm 3.3 a	
	Yes	3	10.0 a	
	Yes	4	13.3 \pm 3.3 a	
	Yes	5	16.7 \pm 12.0 a	
	ABA	No	0	96.7 \pm 5.8 a
		No	2	80.0 \pm 11.5 ab
		No	3	55.0 \pm 5.0 b
		No	4	53.3 \pm 8.8 b
		No	5	66.7 \pm 8.8 ab
Yes		0	0.0 c	
Yes		2	30.0 a	
Yes		3	30.0 \pm 10.0 a	
Yes		4	25.0 \pm 5.0 ab	
Yes		5	10.0 \pm 5.8 bc	
Paclobutrazol		No	0	83.3 \pm 3.3 ab
		No	2	96.7 \pm 3.3 a
		No	3	83.3 \pm 3.3 ab
		No	4	80.0 b
		No	5	73.3 \pm 3.3 b
	Yes	0	0.0 b	
	Yes	2	0.0 b	
	Yes	3	0.0 b	
	Yes	4	36.7 \pm 8.8 a	
	Yes	5	53.3 \pm 8.8 a	

Hairy root beads were dehydrated for different time points and survival rates were recorded 6 weeks after thawing

Each experiment consisted of three repeats with at least 10 hairy roots in each repeat. Different letters indicate significant differences ($P < 0.05$) within one treatment, according to non parametric ANOVA

percentages of hairy roots after freezing. Survival percentages were, however, higher than expected so ABA and paclobutrazol have possibly other effects on the hairy roots, which make them more tolerant for dehydration and freezing.

For *M. lanceolata* as for *M. truncatula* we observed no morphological abnormalities after cryopreservation of the hairy roots (Fig. 1e–h). The growth rates were also the same for non frozen and frozen samples (results not shown).

Discussion

To evaluate and change the production of pharmaceutical important saponins, hairy root lines were established from *M. lanceolata* and *M. truncatula*. Using a standard protocol for *Agrobacterium* mediated transformation we obtained hairy root lines from both species. In order to maintain the original cultures we have developed a cryopreservation protocol.

Because vitrification involves less handling steps in comparison to other cryopreservation techniques (Engelmann 2004), we first tried this method for the cryopreservation of *M. lanceolata* and *M. truncatula* hairy roots. However, the PVS2 solution commonly used for vitrification lead to significant damage of the hairy root tips, also observed for some other tissue and cell structures (Volk et al. 2006). Only very low percentages of the *M. lanceolata* and *M. truncatula* hairy roots survived incubation of 5 min and longer in PVS2, without pretreatment with a loading solution. Roots that were first treated with loading solution seemed to be more resistant to PVS2 solution, as survival percentages decrease from 10–15 min of treatment with PVS2. However, none of the *Maesa* or *Medicago* hairy roots survived freezing in liquid nitrogen after vitrification. In literature, there are only three studies in which vitrification is successfully applied on hairy roots. There is a vitrification procedure described for *Panax ginseng* (Yoshimatsu et al. 1996), *Angelica acutiloba* (Yoshimatsu 2000) and *Atropa belladonna* (Touno et al. 2006) hairy roots. Survival percentages are respectively, 60, 96, and 93%. On the other hand, there are also reports that confirm the negative effect of PVS2 solution on the viability of plant material of some species (Volk et al. 2006; Xue et al. 2008). Xue et al. (2008) tried to cryopreserve hairy roots of three different species using encapsulation-vitrification. For this technique hairy roots were also encapsulated in calcium-alginate beads but were desiccated through treatment with PVS2 solution (Sakai 2000; Xue et al. 2008). No hairy roots of *Gentiana macrophylla* and *Astragalus membranaceus* survived when using the PVS2 solution to vitrify the material. Only 25% of the hairy roots of *Eruca sativa* survived treatment with PVS2 solution (Xue et al. 2008). In view of our own negative results and the few positive results reported in literature on cryopreservation of hairy roots, we concluded that vitrification was not a suitable method to preserve hairy root cultures.

Encapsulation-dehydration is a cryopreservation method that is more labour intensive than vitrification but does not require treatment with cytotoxic compounds (Niino and Sakai 1992). Hairy roots are encapsulated in calcium-alginate beads, precultured for 3 days in medium with increasing sucrose concentration and then dehydrated by the air of a laminar flow cabinet. For *M. lanceolata* hairy

roots we obtained survival percentages of 90% after encapsulation-dehydration. For *M. truncatula* we first encountered the problem that the hairy roots grew too fast and grew out of the bead even before freezing. In this way the root tips were not protected anymore against freezing damage and this resulted in very low percentages of survival. Isolation of smaller root tips was technically too difficult for practical use. So to overcome this problem we added growth inhibitors to the preculture medium, namely ABA and paclobutrazol. Both substances are frequently used as growth retardants in cryopreservation and cold preservation of plant material (Hirata et al. 1998; Hirata et al. 2002; Cha-Um et al. 2007). ABA is also generally known as a plant hormone that plays a role in the acquisition of desiccation tolerance and osmotic stress (Ingram and Bartels 1996; Sharp et al. 2004). ABA induces late-embryogenesis-abundant (*lea*) genes, which encode LEA proteins. These proteins have increased levels during drought and are involved in dehydration tolerance in plants (Ingram and Bartels 1996). Paclobutrazol is a growth retardant that has a negative effect on root growth but it is also an inhibitor of ABA catabolism, so addition of paclobutrazol may increase the endogenous ABA levels in *M. truncatula* hairy roots during desiccation and as a consequence *lea* genes may be induced which might make the hairy roots less susceptible to drought stress (Krizan et al. 2006; Cha-Um et al. 2007). Addition of ABA or paclobutrazol to the preculture media resulted in survival percentages of respectively, 30 and 53%. We believe that these higher percentages are partly due to the reduction of outgrowth and partly due to the higher desiccation tolerance, upon addition of plant growth regulators ABA and paclobutrazol.

There are very few reports on encapsulation-dehydration of hairy roots. The first one was published by Hirata et al. (1998), describing an encapsulation-dehydration protocol for Horseradish hairy root cultures. They obtained survival rates of 60% of the cryopreserved hairy roots. Later, the same group published a report on encapsulation-dehydration of *Vinca minor* hairy roots (Hirata et al. 2002). A survival rate of more than 70% was obtained in this case. Our results show that for *M. lanceolata* 90% and for *M. truncatula* 53% of the hairy roots can be regenerated after cryopreservation. The same protocol is potentially useful for other species, with ABA and paclobutrazol as possible cryoprotectants. In the future we will use the established encapsulation-dehydration protocol routinely for the storage of large numbers of different transgenic root lines of *M. lanceolata* and *M. truncatula*.

Acknowledgements This work was funded by IWT-Flanders; SBO Combiplan, Project No. SBO#040093.

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