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BRIEF COMMUNICATION

Polyamine content during minimal growth storage of *Thymus moroderi* explants

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Abstract

The polyamine (PA) content of *in vitro*-grown explants of *Thymus moroderi* Pau *ex* Martínez has been evaluated during minimal growth storage. The growth restriction was imposed by the combined action of osmotically-active compounds (15 g dm^{-3} sucrose and 15 g dm^{-3} mannitol) present in the Murashige and Skoog medium and the modification of the physical environment of the culture (4 ºC and darkness). In these conditions, cultures were maintained up to 29 weeks without subculture. During this storage period, we analyzed contents of free, perchloric acid (PCA)-soluble and PCA-insoluble conjugated PA. Minimal growth storage brought about an increase in free putrescine (Put) coinciding with a reduction in PCA-soluble conjugated Put occurring during the first weeks of storage. PCA-insoluble conjugated spermidine (Spd) also accumulated in response to storage.

Additional key words: *in vitro* conservation, low temperature, mannitol, putrescine, sorbitol, spermidine, sucrose.

Minimal growth techniques are designed for the short- to medium-term storage of plant germplasm without involving neither freezing facilities or liquid nitrogen handling. In this case, the goal is to modify the physical environment of culture, the medium composition, or both, in order to slow down plant growth and thus to be able to increase the time between consecutive subcultures. The most widely applied strategy is to maintain cultures at low temperature combined with either complete darkness or a reduced irradiance (George 1993). Also, an increased (Gonçalves and Romano 2007) or decreased (Galzy and Compan 1988) sucrose concentration in the culture medium, the addition of sorbitol or mannitol (Harding 1991, Negash *et al*. 2001, Gopal *et al*. 2002, Gonçalves and Romano 2007), or growth modulators like ancymidol (Sarkar *et al*. 2001) or abscisic acid (Negri *et al*. 2000) may lead to effective plant growth reduction. Very scarce information is available on the physiological events occurring within those explants submitted to these culture conditions, which are very close to what we can identify as stressful conditions. To contribute in filling this gap, we have focused on polyamine (PA) content and their possible role in the physiological adjustment of explants to minimal growth storage. In plants PA are involved in many physiological events from DNA replication to fruit ripening (Bouchereau *et al*. 1999) and their involvement in different stress responses is especially relevant (Alcázar *et al*. 2006).

The species selected for this work is *Thymus moroderi* Pau *ex* Martínez (*Lamiaceae*). It is endemic to south-eastern Spain, highly appreciated in the liqueur industry and folk medicine and considered as near threatened according to the IUCN categories. We have recently published several long-term cryopreservation-based approaches for this species (Marco-Medina *et al*. 2010a,b). However, a medium-term storage protocol for this species is lacking. Therefore, the goal of this work was to design an effective conservation protocol for *Thymus moroderi*, based in minimal-growth techniques, and to follow the changes in polyamine content during minimal growth storage in order to gain insights into the physiological adjustment occurring in explants during

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Abbreviations: Cad - cadaverine; Dap - diaminopropane; FMOC - N-9-fluorenylmethylchloroformiate; MS - Murashige and Skoog; PA - polyamine; PCA - perchloric acid; Put - putrescine; Spd - spermidine; Spm - spermine.

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this type of storage.

Explants for minimal growth experiments were shoot tips of 1.5 cm in length each comprising two-three nodes and obtained from healthy *in vitro* growing plantlets of *Thymus moroderi*. These plantlets were cultured in Murashige and Skoog (1962; MS) medium supplemented with 0.08 M sucrose, 7 g dm^{-3} agar and pH adjusted to 5.8 (basal medium) and maintained at 24 ± 1 °C under a 16-h photoperiod, with an irradiance of 42 μmol m^{-1} s^{-1} (standard conditions). The potential of low temperature (4 ± 1 °C) and darkness (minimal growth conditions) to restrict explant growth was tested. Different osmotic agents were applied in the MS medium: sucrose (ranging from 15 to 30 g dm^{-3}) in combination with either mannitol or sorbitol (in both cases ranging from 15 to 40 g dm^{-3}). Explants were maintained in these media for different times before being transferred to basal medium and standard conditions. The effectiveness of the minimal growth treatments was assessed by measuring explant length increase during storage and after being transferred to standard conditions.

Individual PA content from different physiological pools (free, PCA-soluble conjugated and PCA-insoluble conjugated PA) existing in leaf tissue was analyzed. The extraction and analysis protocols were essentially those outlined in Sharma and Rajam (1995) with minor modifications. For PA analysis, samples were firstly filtered through 0.45 μm filters and derivatized with N-9-fluorenylmethylchloroformiate (FMOC) to form fluorescent complexes (Yokota *et al*. 1994), before being separated by high performance liquid chromatography (HPLC) and identified by comparing their retention time with that of authentic PA (*Sigma-Aldrich*, St. Louis, USA). PA contents were calculated using 1,6-diaminohexane as internal standard.

Statistical analyses were performed with *SPSS 15.0* (*SPSS Inc.*, Chicago, USA) for *Windows*. The significant differences between means of explant length were assessed by analysis of variance (*ANOVA*) and Duncan's multiple range test ($P < 0.05$), while differences in PA analysis were evaluated by the *t*-test for independent samples at 5 % level of probability.

In a first set of experiments, different combinations of sucrose plus mannitol or sorbitol associated with low temperature and darkness were tested. All media assayed reached an acceptable restriction of growth after 3 weeks under minimal growth conditions (Table 1). Four weeks after being transferred to standard conditions and basal medium, all treatments, except 30 g dm^{-3} mannitol, allowed explant growth reactivation. In view of these results, we choose as minimal growth medium MS medium supplemented with 15 g dm^{-3} sucrose and 15 g dm^{-3} mannitol for the subsequent experiments. In order to determine whether this response was due to the combined presence of both osmotic agents or to the isolated action of just one of them, mannitol and sucrose at 15 g dm^{-3} were tested again in combination and separately under minimal growth conditions. The results

Table 1. Effect of different storage conditions on explant length increase [cm]. Explants stored under minimal growth conditions and cultured in different media were measured after 3 weeks storage and further 4-weeks on basal medium and in standard conditions. Means ± SD, $n = 12$. Values with the same letter within the column are not significantly different according to Duncan's multiple range test ($P < 0.05$).

Medium [g dm^{-3}]	3 weeks	3 + 4 weeks
15 sucrose + 15 mannitol	0.14 ± 0.12 a	1.36 ± 0.39 a
30 mannitol	0.02 ± 0.07 c	0.07 ± 0.95 c
20 sucrose + 40 mannitol	0.01 ± 0.08 c	0.88 ± 0.41 b
30 sucrose + 30 mannitol	0.12 ± 0.14 ab	0.91 ± 0.82 ab
15 sucrose + 15 sorbitol	0.16 ± 0.11 a	0.88 ± 0.44 b
30 sorbitol	0.08 ± 0.09 abc	0.88 ± 0.46 b
20 sucrose + 40 sorbitol	0.03 ± 0.06 c	0.95 ± 0.40 ab
30 sucrose + 30 sorbitol	0.05 ± 0.05 bc	1.05 ± 0.43 ab

obtained showed that after being transferred to basal medium and standard conditions, explants stored in MS supplemented with both mannitol and sucrose showed the highest length increase and also formed visually normal rooted plantlets (data not shown). Consequently this combination was considered as the minimal growth medium of choice for the storage of *Thymus moroderi* explants, and a subsequent experiment was carried out to assess the ability of minimal growth medium combined with minimal growth conditions to restrict explant growth for an extended period of time. The results (Table 2) showed that these conditions effectively restricted explant growth. However, from the 12th week those explants stored in minimal growth medium and standard conditions started to show symptoms of dehydration that prevented them to survive for longer period of times under these conditions. Only those explants cultured in minimal growth medium and stored under minimal growth conditions remained alive after 29 weeks. These explants did not root nor multiply during this period but upon being transferred to basal medium and standard conditions explants were able to resume growth and 80 % of them rooted after 8 weeks. To reinforce the need for simultaneous applying minimal growth medium and minimal growth conditions to successfully store *T. moroderi* explants, we also tested to store explants in basal medium and minimal growth conditions but after 29 weeks 100 % of explants dried out (data not shown). In contrast, a minimal growth medium like that used for *T. moroderi* was successfully employed in *Vanilla* but in combination with room temperature (Divakaran *et al*. 2006). These results reflect the complex interaction operating among the parameters of a minimal growth protocol for each particular species. For instance, explants of *Quercus suber* stored at 5 ºC and darkness gave a 50 % survival after 2 years without subculturing. This percentage, however, dramatically decreased when storage was carried out under irradiance (Romano and Martins-Louçao 1999). Cultures of *Drosophyllum*

lusitanicum were conserved for 8 months in medium containing high concentration of sucrose and mannitol at 5 ºC in the dark (Gonçalves and Romano 2007). The apparent lack of a general rule for the combination of temperature, osmotic agents, light and darkness for a given species obliges the researcher to optimize them in each case. We have found that the combined presence of minimal growth conditions and a minimal growth medium was required for the effective conservation of *T. moroderi* cultures.

But, what changes do operate in explants to adapt to these stressful conditions? We have focused on PA content because of their involvement in many developmental processes as well as in stress responses in plants.

Free putrescine (Put), spermidine (Spd) and spermine (Spm) were found in leaves of *T. moroderi* explants during minimal growth storage. However, only Put and Spd are presented here (Table 2) because the content of Spm was frequently below the detection limit of the HPLC and therefore gave inconsistent results. Put content increased 2.7-fold one week after the onset of storage and decline thereafter until reaching its lowest value at the end of the cold storage period studied (29 weeks, Table 2). A similar trend was recorded in explants maintained in minimal growth medium and standard conditions as in controls. In both cases, Put peaked at 48 h of storage but the highest concentration reached was only double than initial. It is remarkable that at week 8 of storage, the free Put content found in leaves of *T. moroderi* was essentially the same irrespective to the storage conditions applied (Table 2).

Free Spd showed a similar trend irrespective to the storage conditions applied and opposite to that of Put. Spd initially decreased during the first 1 - 2 weeks of storage and slightly increased since then (Table 2), reaching in the case of explants stored under minimal growth conditions and in minimal growth medium its highest content at the end of the cold storage period (Table 2).

The increase in free Put in response to low temperature stress has been reported by different authors. In mango, increase in Put was induced by low temperature but it did not prevent chilling injury (Nair and Singh 2004). In *Oryza sativa* roots, an increase in Put was observed 3 d after the exposure to low temperature, and was related with the ability of tolerating chilling stress because the application of PA synthesis inhibitors produced a decrease in the recovery of root growth after chilling (Lee 1997). Liu *et al.* (2011) found in *Vitis vinifera* seedlings exposed to NaCl an increase in free Put during the first days of exposure to this stress. The study of the *Arabidopsis* metabolome after temperature stress revealed an increase of different metabolites including Put during the acquisition of both chilling and heat

Table 2. Effect of the different storage conditions assayed on explant length increase [cm] and polyamine content [nmol g^{-1}(d.m.)] of *T. moroderi* explants. Means ± SE, $n = 3$. Values followed by the same letter within the same parameter and period of time are not significantly different ($P = 0.05$) according to *t*-test (polyamine content). With refers to length increase, values followed by the same letter are not significantly different according to Duncan's multiple range test ($P < 0.05$). PCA - perchloric acid; Cad - cadaverine; Dap - diaminopropane; MG - minimal growth medium; MC - minimal growth conditions; SC - standard conditions; control - basal medium + standard conditions.

Parameter	Treatment	0 h	48 h	1 week	2 weeks	4 weeks	8 weeks	29 weeks	29+8 weeks
Length increase	MG+MC	-	0.05±0.02e	0.10±0.01de	0.16±0.02de	0.14±0.03de	0.27±0.06de	0.48±0.02	0.82±0.07
	MG+SC	-	0.01±0.00e	0.09±0.03de	0.13±0.03de	0.14±0.03de	0.20±0.04de	-	-
	control	-	0.13±0.02e	0.46±0.06d	1.07±0.03c	2.81±0.01b	5.26± 0.06a	-	-
Free Put	MG+MC	1317±259	2970± 900a	3587±184a	3549±133a	2438±124a	588±94a	146±12	1141±249
	MG+SC	731±54	1398±301a	970±55b	769±101b	899±221b	487±71a	-	-
	control	752±8	1553±304a	796±257b	566±102b	907±36b	332±38a	-	-
Free Spd	MG+MC	898±92	536±134ab	452±120a	329±44b	579±73a	708±95a	907±33	647±94
	MG+SC	685±124	465±98b	302±29a	320±38b	453±11a	488±143a	-	-
	control	1015±148	903±96a	455±107a	659±108a	702±74a	668±129a	-	-
PCA-soluble Put	MG+MC	981±468	806±580b	631±562a	353±342b	215±372b	2027±562a	2234±374	1477±271
	MG+SC	1693±267	2482±315a	1275±388a	1242±107a	1165±262a	2230±197a	-	-
	control	2135±163	1797±258ab	1359±71a	1335±223a	1949±336a	1593±200a	-	-
PCA-insoluble Dap	MG+MC	254±35	516±65a	896±283a	572±104a	295±49b	739±112a	551±201	504±204
	MG+SC	616±227	803±353a	544±156a	875±558a	388±122ab	707±393a	-	-
	control	385±103	567±259a	1130±529a	373±84a	580±15a	382±20a	-	-
PCA-insoluble Cad	MG+MC	521±108	846±110a	1260±426a	840±110a	381±59a	1254±19a	919±268	896±160
	MG+SC	980±125	1564±721ab	596±199a	531±63a	1090±373a	617±166b	-	-
	control	934±198	357±60b	740±335a	525±181a	1181±186a	419±251ab	-	-
PCA-insoluble Spd	MG+MC	44±44	78±8	97±5	78±10a	91±24a	178±46a	18±9	11±9
	MG+SC	134±19	-	124±12	83±22ab	102±8a	124±25a	-	-
	control	25±12	-	-	0.7±0.7b	12±4b	29±17b	-	-

tolerance (Kaplan *et al*. 2004), coinciding with results obtained also in maize (Gao *et al*. 2009). Recently, cold stress was shown to up-regulate the expression of arginine decarboxylase and S-adenosylmethionine decarboxylase, both involved in Put synthesis (Oufir *et al*. 2008).

It is known that PA do not only exist as free form in living cells but bound with different cell components. We have analyzed these other PA fractions according to their solubility in perchloric acid (PCA). In PCA-soluble conjugated PA fraction only Put was detected. PCA-soluble conjugated Put decreased steadily during the first 4 weeks of storage in minimal growth medium treatments and, in explants stored in minimal growth conditions, the drop in this amine was more pronounced. After 8 weeks, Put content levelled in the three storage conditions assayed (Table 2).

Coinciding with the increase in free Put a decrease in PCA-soluble conjugated Put was observed under minimal growth conditions. This suggests that the sudden increase in free Put may be at least partially due to the release from conjugated Put, although a direct effect of low temperature on the enzymes involved in Put biosynthesis cannot be discarded (Oufir *et al.* 2008). A similar trend has been described in roots of non-mycorrhizal seedlings of *Pinus sylvestris* in response to daylength shortening (Sarjala and Taulavuori 2004) and in transgenic and wild type plants of *Arabidopsis* subjected to chilling stress (Kasukabe *et al.* 2004). After a month under minimal growth conditions, free and PCA-soluble conjugated Put inverted their trend, which may be interpreted as a symptom of the adaptation of explants to the new storage conditions. Interestingly, when explants were transferred to standard conditions and basal medium again the pair free Put/PCA-soluble conjugated Put reacted in response to the new culture conditions. These findings reinforce the role of Put, both in free and in conjugated form, as a physiological detector of stress in explants.

When analyzed the PCA-insoluble conjugated PA fraction, diaminopropane (Dap), cadaverine (Cad) and Spd were detected (Table 2). The presence of PCA-insoluble Dap and Cad was remarkable, although in both cases there were no significant differences among storage conditions. Dap is the result of Spd and Spm oxidation and it has been found to accumulate under osmotic stress (Aziz *et al*. 1997). Cad has been related with the ability to grow under extreme conditions (Carrizo *et al*. 2001) or osmotic stress (Aziz *et al*. 1997), although the results reported in these two works were referred only to the free fraction. The fact that in our experiments these two amines were not detected in free form may suggest a specific role for these conjugates in explant adaptation to stress, a hypothesis which deserves further research. Spd was detected at lower concentrations than the other two conjugated amines, especially in control plants in which it was detected only from week 2 (Table 2). PCA-insoluble Spd was detected at higher concentrations when mannitol was present in the medium irrespective to whether storage was at low or room temperature (Table 2). This fact and the pattern found in this Spd form may suggest a protective role of PCA-insoluble conjugated Spd under stress conditions. Such protective role has already been proposed for the free form of Spd in maize exposed to low temperature (Gao *et al*. 2009) as well as in *Vitis vinifera* roots under salinity stress (Upreti and Murti 2010).

In summary, we have developed a minimal growth storage protocol for *Thymus moroderi* based in the growth restriction imposed by the combined action of osmotically-active compounds present in the culture medium (sucrose and mannitol) and the modification of the physical environment (4 ºC and darkness). In these conditions, explants may survive at least 29 weeks without subculturing. Three main events have been registered regarding PA content during minimal growth storage: a sudden and drastic increase in free Put during the first week of storage; a concomitant drop in PCA-soluble conjugated Put that extended during the first four weeks and a steady increase in PCA-insoluble conjugated Spd. In the early adaptation of explants to these storage conditions the pair free/PCA-soluble conjugated Put seems to play a pivotal role.

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