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Micropropagation of the endangered desert shrub *Haloxylon persicum*

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Abstract

The endangered palatable species *Haloxylon persicum* (Bunge ex Boiss & Buhse) is a favored plant to stabilize sand dunes, prevent soil desertification, conserve water and soil, and improve environmental conditions. To develop an efficient and reproducible plant regeneration system, single node segments of 1-month-old *H. persicum* seedlings were cultured on modified Murashige and Skoog medium. Plant growth regulators significantly affected bud sprouting and callus and adventitious shoot formation. After 3 months of culture, 10–98% of the explants had sprouted buds, whereas the percentage of explants that developed calli was 0–93%. Kinetin (Kin) was more appropriate for bud sprouting and the development of adventitious buds than 6-benzylaminopurine (BA). Shoot-tip necrosis (STN) was observed after the first subculture in baby jars and became more severe after the transfer to ventilated boxes. The explants cultured on medium supplemented with BA showed severe STN compared with media containing Kin. When 10 µM Kin. was the sole plant growth regulator in the medium, the developing calli gave rise to multiple shoot buds and was maintained for 6 months without losing its regenerative ability. A half-strength medium supplemented with 10 µM Kin., 100 or 200 mM boron, and 2.5 or 5 mM calcium prohibited STN. When the half-strength medium was supplemented with 100 mM boron and 2.5 mM calcium, 60% of the explants produced sprouted buds, with an average number of 4.5 shoots longer than 3 cm per explant, and no STN was observed. Furthermore, 20% of these shoots developed roots on half-strength medium supplemented with 30 µM indoleacetic acid (IAA) and were adapted to greenhouse conditions.

Keywords: Boron; Calcium; Culture ventilation; Shoot-tip necrosis; White saxual.
Abbreviations: BA- 6-Benzyladenine, IAA- indoleacetic acid, IBA- Indole-3-butyric acid, Kinetin – Kin, MS- Murashige and Skoog basal medium, MSM- Modified Murashige and Skoog basal medium, PGRs- Plant growth regulators, STN- Shoot-tip necrosis.

Introduction

The white saxual multipurpose shrub *Haloxylon persicum* (Bunge ex Boiss & Buhse), which belongs to the Amaranthaceae family, is a typical desert-inhabiting small tree. The plants grow to 2–4 m in height, have a large underground root system and photosynthesize through small photosynthetic branches (Ruan et al., 2006). The species is scattered throughout northwestern China and northern Russia (Hadi and Kharazipour, 2008); in the Middle East, the species is distributed mainly in the Sinai Peninsula, Egypt, and central and northwestern Saudi Arabia on the Arabian Peninsula (Zohary, 1973). The species is termed a super-xerophyte because of its extreme drought tolerance, which enables it to survive in prohibitive environments. Therefore, this species has been particularly favored as a pioneer plant to stabilize sand dunes, prevent soil desertification, conserve water and soil, and improve the environment for wildlife under conditions that few large shrubs can tolerate. *H. persicum* is also a palatable species for livestock and an excellent plant material to investigate the physiological characteristics of stress adaptation (Zhang, 2002). However, overgrazing and the misuse of rangelands have caused the disappearance of this useful species and the dominance of unpalatable plants in many areas of its distribution. Hamadeh (2005) emphasized that western Asia is losing such important species as *H. persicum*. The freshly harvested seed of this species is reported to germinate well, but the seeds rapidly lose viability (Birnbaum et al., 2000). Micropropagation has many advantages over conventional propagation techniques and is applied for the mass production of genetically identical, physiologically uniform, developmentally normal and pathogen-free plantlets, which can be acclimatized in a short time period and at a lower cost (Rathore et al., 2005). Furthermore, micropropagation holds promise for the mass multiplication of valuable woody species that are on the verge of extinction (Harry and Thorpe, 1994). Al-Khalifah (2004) investigated the in vitro culture of *H. persicum* and concluded that further study should be conducted to address the problems of a low percentage of organogenesis and lack of shoot elongation. Indeed, there has been progress in the micropropagation of other Chenopodiaceae species, a family now included in Amaranthaceae, such as the important woody fodder plants of genera *Atriplex* and *Kochia*. Birnbaum (2001) cultured *H. aphyllum* cotyledonary nodes on different media supplemented with 6-benzyladenine (BA). Although Murashige and Skoog (MS; 1962) basal medium was optimal for the micropropagation, with the highest number of shoots (5 per explants) achieved on MS medium supplemented with 11 µM BA, the longest shoots were achieved on a hormone-free medium. A hard, compact callus was developed from the base of the explants, and 50% of the shoots produced roots when incubated on a medium supplemented with 2.89 or 28.55 µM IAA before the transfer

to a medium lacking plant growth regulators. Shoot-tip necrosis (STN), which is a physiological disorder, whereby the apical shoot initially becomes brown and subsequently dies, commonly affects in vitro cultures of a wide range of plants (McCown and Sellmer, 1987). Bairu et al. (2009a;b) reviewed the factors that cause this phenomenon during micropropagation and concluded that numerous environmental and nutritional factors are involved in STN. Among these factors, improving the culture ventilation (Thomas, 2000; Jain et al., 2009) and avoiding a deficiency of some nutrients, such as calcium and boron (Singha et al., 1990; Xing et al., 1997; Abdulnour et al., 2000; Bairu et al., 2009b), can be applied to overcome STN. Therefore, the purpose of the present study was to develop an efficient and reproducible plant regeneration system for *H. persicum*.

Results and discussion

Direct and indirect regeneration

Sprouted axillary buds developed after 3 months of culture. Kin., BA and indole-3-butyric acid (IBA) had significant affects on all of the investigated traits, and there was a significant interaction between these plant growth regulators (PGRs). The percentage of explants with sprouted axillary buds varied significantly ($P<0.05$) among the treatments (Table 1). In general, higher percentages of explants with at least two sprouted buds were obtained on the medium supplemented with Kin. Almost all of the explants cultured on the medium supplemented with 10 µM Kin. sprouted buds, but this value was reduced to 70% and 37% on the media supplemented with 5 and 10 µM IBA, respectively (Table 1). Callus growth was initiated from the base of some explants, and increased on some media, completely covering the explants. The callus size varied between the treatments: the calli were larger on the media that contained BA in comparison to kinetin (Table 1). The calli that developed from the explants cultured on a medium with zero or 5 µM IBA and Kin. were the smallest (<1 cm in diameter), whereas the calli that developed on the medium with 10 mM IBA and BA were the largest (>2.0 cm). Meristematic regions were observed on some of the developing calli, and multiple adventitious shoots buds were initiated (Table 1). In addition, the percentage of adventitious shoots that regenerated from the developing calli varied significantly with the PGR in the medium. Kin. was found to be more suitable for *H. persicum* organogenesis than BA because 24–80% of the developing calli had adventitious buds compared with 0–17% on the medium supplemented with BA (depending on the concentration of IBA in the medium). This pronounced effect of the plant growth regulator (PGR) on the in vitro culture of *H. persicum* is comparable to the results obtained with *H. aphyllum* (Birnbaum 2001). Indeed, much evidence confirms that PGRs play a vital role on in vitro plant growth and development, and the actions of auxins and cytokinins as promoters of callus formation and adventitious shoot development are well known. However, these effects depend on the type and concentration of the auxin and cytokinin and the ratio between them (George et al. 2008). Some explants cultured on auxin-free medium developed calli and adventitious shoots; moreover, calli that originated on a medium supplemented with Kin. were maintained on that medium for approximately 6 months without losing their organogenetic ability. Some tissues, organs or cell strains are able to grow without the incorporation of auxin in the medium; such cultures are termed auxin autonomous or auxin habituated (Machakova et al. 2008). Syono and Furuya (1971) reported that 0.46–5 µM Kin. appears to increase the natural auxin content of the tissues but appears not to cause auxin habituation. The *H. persicum* seeds germinated on MSM medium within 2 days of culturing, and the seedlings were maintained on this medium for 3 months without showing any symptoms of STN. In addition, the nodal segment cuttings were cultured without rooting and were subcultured on PGR-free MSM for several months without the development of STN. No STN symptoms were observed during the first culture, whereas STN was observed after approximately 2 weeks of the first subculture (Fig. 1a). The symptoms were first apparent on the terminal shoot tips and expanded to affect the axillary shoots, particularly the one closest to the shoot tip. The death of the shoot tips enhanced shoot-bud sprouting, and the sprout later died. More severe STN was observed on the media supplemented with BA compared with the media that contained Kin. It was interesting observing STN regardless, callus development or its size furthermore; the explants cultured on the medium supplemented with 10 µM Kin. as the sole PGR developed roots but suffered STN (Fig. 1b). On this medium, 60% of the explants developed roots but also experienced severe STN and the enhanced growth of adventitious buds (Fig. 1c). Despite considerable research on the relationship between STN phenomena and PGRs, there is no definitive explanation for their in vitro effects (Bauir et al. 2009b). In some instances, the type and concentration of both cytokinins and auxins could affect the presence and severity of STN. In the present study, normal growth of the *H. persicum* seedlings and shoot cuttings (without rooting) was obtained on PGR-free media, and the presence of STN was noted on media supplemented with different combinations of PGRs (data not presented). In addition, calli and roots formed from the bases of the shoots that showed STN, indicating that the PGR balance was not the main cause of the observed STN. The shoots that developed on the medium supplemented with 10 µM Kin. produced many long roots, and STN coincided with the death of the main shoot and the development of multiple adventitious shoots after 8 months of growth. Those observations conflict with those of Kataeva et al. (1991) and Piagnani et al. (1996) who suggested that the elimination or reduction of the concentration of cytokinin to a very low level because of their anti-rooting effect, is the cause of STN. In addition, our results are the opposite of those of Bairu et al. (2009b) who considered that the lack of roots, which are the main sites of cytokinin biosynthesis, could increase STN. However, Bairu et al. (2009a) concluded that the type of cytokinin plays a significant role in STN. Therefore, our results might indicate that the concentrations of BA and Kin. used were not effective in avoiding STN in *H. persicum*. The calli associated with the adventitious shoots that developed on the medium supplemented with 10 µM Kin. (Fig. 1b) were trimmed from the shoot bases and subcultured on fresh media. Although these calli were maintained for 6 months, the shoot buds did not elongate beyond approximately 0.4 cm long (Fig. 2a) without the development of STN symptoms. Because the aim of this study was to optimize a protocol for the direct regeneration of *H. persicum*, this culture was not considered in the ensuing investigation.

Overcoming shoot-tip necrosis

The *H. persicum* cultures were transferred from the baby jars to full-gas microboxes to provide a high ventilation rate and a

Table 1. Effect of plant growth regulators on the in vitro culture of *H. persicum* single-node explants after 3 months of culture on shoot induction medium (baby jars were used for the first 2 months, and full-gas microboxes were used thereafter)

PGR (µM)		Axillary buds	Callus (%)†	Callus size	Callus with adventitious	Shoot tip necrosis
IBA	BA	sprouting (%)[1†]		(cm)	shoots (%)[2]†	(%)†
0	5	25±2d	24±4h	1–1.5	17±2e	100
	10	36±3d	27±3g	1.5–2.0	15±1e	100
5	5	10±1f	0	-	0	100
	10	20±2e	0	-	0	100
10	5	10±3f	93±3a	>2.0	7±2f	90
	10	25±2d	29±2 g	>2.0	10±3f	94
	Kinetin					
0	5	58±4c	38±3de	< 1	45±4c	83
	10	98±2a	80±4b	< 1	80±5a	80
5	5	58±3c	57±5c	< 1	57±3b	95
	10	70±4b	45±4d	< 1	45±4c	86
10	5	28±3d	35±3ef	1.5–2.0	24±2d	92
	10	37±4d	30±4fg	1.5–2.0	30±3d	80

[1]Each presented value was calculated based on 30 explants ±SE; [2]based on observations of more than two axillary sprouted buds. † The percentage frequencies not followed by the same letter differ significantly at P=0.05 using the Chi-squared test**.**

consequent low humidity. Additional green calli and indirect adventitious buds were initiated from the base of the explants, and, in some instances, the calli completely covered the original explants. However, STN was observed in all of the cultures, indicating that ventilation had no notable effect on the occurrence of STN in *H. persicum*. This result is contrary to the hypothesis that an insufficient supply of Ca occurs because of the limited uptake or transport that is caused by the absence of transpiration as a result of the high humidity in a standard culture vessel (Bairu et al., 2009b). Thomas (2000) and Jain et al. (2009) found that improved vessel ventilation reduced the frequency of STN in different woody species. Thus, the conflicting results obtained for *H. persicum* may be the result of a genotypic effect. Raising the concentration of Ca and B in the growth medium significantly reduced the frequency of STN and additional callus formation but enhanced shoot elongation (Table 2) (Fig. 2b). In addition, there were significant interactions among Ca, B and the medium strength. Interestingly, unlike a previous experiment and regardless of the medium composition, few of the explants developed small-sized calli on the base of the explants. The percentage of explants with sprouted buds was significantly (*P<0.05*) decreased depending on the strength of the MSM medium and the concentration of Ca and B (Table 2). At the same concentration of Ca and B, the half-strength medium reduced the number of sprouted buds compared with the full-strength medium (Table 2). The medium supplemented with 200 mM B and 2.5 or 5 mM Ca had the highest percentage of explants, with the sprouted buds on the full-strength medium being higher than the half-strength medium. The number of developing shoots (>1 cm in length) varied significantly (*P<0.05*) among the different treatments (Table 2). The highest numbers of developing shoots (5.07 and 4.85) were obtained on the full-strength media supplemented with 5 mM Ca and 5 mM Ca + 100 mM B, respectively. Nevertheless, no general trend for the effect of Ca and B on the number of developing shoots was apparent. Although a reduction of the non-organic content of the growth medium, in addition to increased Ca and B concentrations, significantly reduced the frequency of STN, symptoms were still observed on the full-strength media (regardless of the Ca and B concentration) and on the B-free half-strength media. All of the explants cultured on the half-strength medium supplemented with Ca

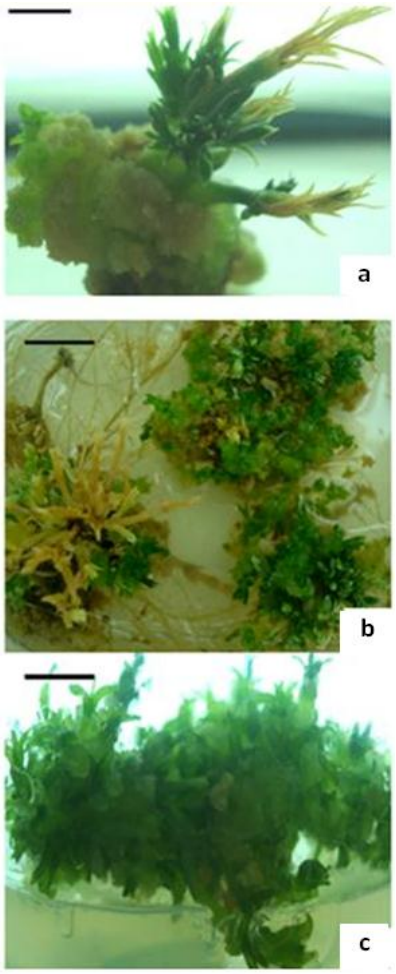

Fig 1. *Haloxylon persicum* explants showing shoot-tip necrosis and developing calli with adventitious buds on a medium supplemented with 5 µM BA and 10 µM IBA (**a**), explants with roots and severe shoot-tip necrosis on a medium supplemented with 10 µM Kin. (**b**), and multiple adventitious shoots formed from developing calli without shoot-tip necrosis on a medium supplemented with 10 µM Kin. (**c**). Scale bar = 1 cm.

Table 2. Effect of the medium strength and calcium and boron concentrations on shoot tip necrosis, regeneration and rooting of developing shoots of *H. persicum* single-node explants on rooting medium

B/Ca (mM)		Medium	Explants with	No. of	Shoot tip necrosis of the	Rooting
B	Ca	strength	sprouting buds (%)[1†]	shoots/explant[2††]	main shoot (%)[†]	(%)[3†]
0	2.5	Full	75±4bc	3.67±0.21de	74±5b	3±0.5fg
	5		77±2b	5.07±0.34a	70±4b	2±0.5 g
100	2.5		70±4c	3.85±0.22cd	68±3b	5±1.0f
	5		80±3b	4.85±0.14a	72±4b	8±0.9eg
200	2.5		90±5a	4.15±0.08bc	81±5a	5±0.2f
	5		85±5a	2.50±0.114 h	82±2a	7±0.8ef
0	2.5	Half	50±4e	2.8±0.10fg	65±3bc	25±2.0b
	5		58±2de	3.5±0.13de	59±2c	35±3.5a
100	2.5		60±3d	4.5±0.10ab	0	20±3.0c
	5		60±4d	3.1±0.11fg	0	10±1.8e
200	2.5		75±3bc	3.0±0.09fg	0	15±1.4d
	5		73±5bc	3.3±0.11ef	0	10±1.2e

[1]Each presented value was calculated based on 30 explants ±SE; [2]based on observations of more than two axillary sprouted buds; [3]shoots longer than 3 cm were separated and cultured for 2 months on half-strength MSM medium supplemented with 30 µM IAA for root induction. [†]The percentage frequencies not followed by the same letter differ significantly at P=0.05 using the Chi-squared test.[††]Values not followed by the same letter differ significantly at P=0.05 using Tukey's test.

and B showed shoot elongation but no STN symptoms (Fig. 2c). A shortage of Ca during the multiplication stage is associated with tip necrosis in many woody species (Sha et al., 1985; Singha et al., 1990). Calcium is important for some of the changes that could contribute to the physiological aging associated with STN, as Ca deficiency causes serious damage to the plant cell ultrastructure, resulting in the rapid deterioration of metabolically active tissues. Ca mediates the effect of auxins and cytokinins, however reports about the effect of Ca on STN lack consistency with regard to the plant species (for a review, see Bairu et al., 2009a). The addition of Ca to the full-strength medium was more effective against STN when the medium contained a higher concentration of B compared with that of MS (Table 2). These results were similar to those of Abousalim and Mantell (1994) with respect to *Pistachia* plants. Boron is an essential element for the maintenance of meristematic activity, most likely because it is involved in the synthesis of N bases, particularly uracil, which is required for RNA synthesis (Mengel and Kirkby, 1982). Abdulnour et al. (2000) reported that the B and Ca concentrations influence the uptake of each another, therefore it is important to optimize the level of B and Ca in the culture medium while addressing the problem of STN. Bariu et al. (2009) added that the effects of B and Ca on one another are also affected by other components of the medium, such as the PGRs. A clear reduction in the frequency of STN compared with the previous experiment using MS medium with the typical concentration of Ca and B was observed, particularly for the half-strength medium. The symptoms of STN were completely eliminated under our micropropagation conditions by the addition of B and Ca to the half-strength medium. The pronounced effect of the concentration of non-organic constituents on cultured explants, together with the different stages of micropropagation, are well documented (George et al., 2008). The significantly positive effect of a half-strength medium for the prevention of STN in *H. persicum* was similar to the findings of previous investigations in *Harpagophytum procumbens* (Bairu et al., 2009b; Jain et al., 2009). Bairu et al. (2009a) considered that there is a general tendency to attribute the occurrence of STN to the high salt concentration of MS. Boron appears to have a vital role in the prevention of STN, which was observed on the B-free half-strength medium, regardless of the concentration of Ca. Some *H. persicum* explants developed

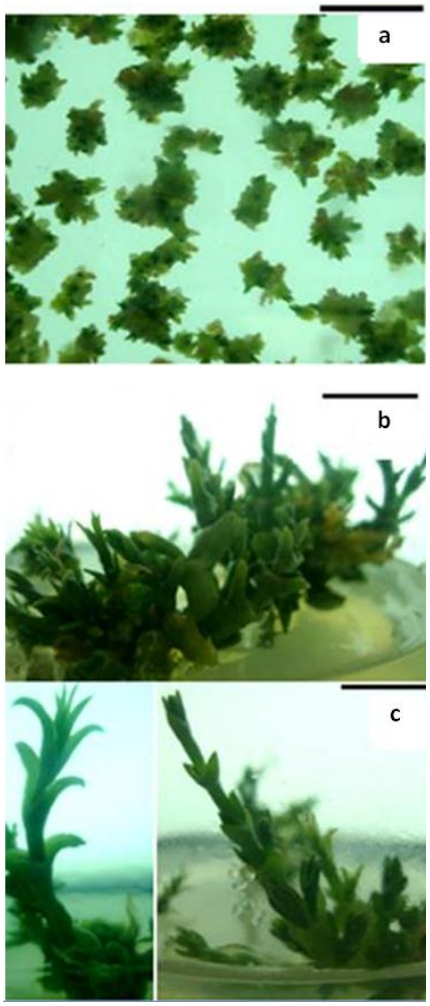

Fig 2. *Haloxylon persicum* calli showing adventitious shoots after 6 months of subculture on a medium supplemented with 10 µM Kin. (**a**), explants cultured on a half-strength growth medium supplemented with 10 µM Kin.,100 mM B and 2.5 mM Ca showing no shoot-tip necrosis (**b**), and shoot elongation on a half-strength growth medium supplemented with 30 µM IAA, 100 mM B and 2.5 mM Ca (**c**). Scale bar = 1 cm.

roots on both types of growth media, but the rooting percentage varied significantly (P<0.05) among the treatments (Table 2). On the half-strength MSM medium, 10–35% of the cultured explants developed roots, whereas this value was reduced to 2–8% on the full-strength medium. The highest percentage of rooting was achieved on the half-strength medium supplemented with 5 mM Ca and 0 mM B (Table 2). The shoots longer than 2 cm were excised and cultured on the half-strength medium supplemented with 100 mM B and 2.5 mM Ca for root induction. Subsequently, the rooted shoots were found to be adapted to greenhouse conditions. In conclusion, an efficient protocol for plantlet regeneration from single nodes of *H. persicum* was developed using a half-strength modified MS medium supplemented with 10 µM Kin., 100 mM boron and 2.5 mM calcium. However, rooting of the developing shoots was achieved on media supplemented with 30 µM indoleacetic acid (IAA). This protocol may permit in vitro mass propagation in efforts to combat desertification with elite clones and the conservation of this threatened desert shrub.

Material and methods

Plant material

The freshly collected seeds of *Haloxylon persicum* were kindly provided by the Research Center for the Development of Range and Animal Wealth, Aljouf, Saudi Arabia. The seed wings were removed before washing under running tap water for 1 h. The seeds were surface sterilized with 96% (v/v) ethanol for 5 s and with 20% (v/v) commercial Clorox bleach (Clorox Co., Jeddah, Saudi Arabia) for 15 min, followed by several rinses with sterile distilled water. The surface-sterilized seeds were soaked in sterilized distilled water for 4 h and germinated on modified MS medium. The composition of this medium was MS medium supplemented with additional 5.20 mM phosphate (provided as $NaH_2PO_4.2H_2O$), 100 mg l^{-1} adenine sulfate, 80 mg l^{-1} *myo*-inositol, 0.5 mg l^{-1} thiamine-HCl and 3% (w/v) sucrose. Throughout the experiment, the pH of the media was adjusted to 5.8 prior to autoclaving at 121°C for 15 min at 105 kPa. All of the cultures were incubated at 22±1ºC under a 16 h photoperiod, with an irradiance of 50 µmol m^{-2} s^{-1} provided by Phillips TLD 50 W/84 HF fluorescent tubes. All of the media were solidified with 8 g l^{-1} agar (BDH, Poole, UK). All of the explants were transferred to fresh media every 4 weeks.

Plant proliferation

One month after germination, the roots, cotyledon nodes and shoot tips of the seedling were discarded, and the remainder of the seedling was divided into two single-node segments, each of approximately 1.5 cm. The explants were cultured on shoot proliferation medium, which consisted of MSM medium supplemented with 0, 5 or 10 µM indole-3-butaric acid (IBA) + 5 or 10 µM 6-benzyladenine (BA) or 5 or 10 µM Kin. The medium was dispensed into 250 ml baby jars, with eight baby-jar replicates, each with five explants, for each treatment.

Overcoming STN

Shoot-tip necrosis was observed on the newly proliferated shoots for almost all of the cultures after the first subculture; thus, all of the explants from the previous culture were transferred to a full-gas microbox with green filters (Eco 2 NV, Geraardsbergen, Belgium), ensuring 62.87 air replacements per day. Each box contained 50 ml growth medium with eight explants. The size of calli that developed on the base of the explants was scored visually, and the calli that developed on the medium supplemented with 10 µM Kin. were trimmed and divided into small pieces (approximately 0.5 cm in diameter) and subcultured six times at 4 week intervals onto fresh media dispensed into 9 cm Petri dishes. To prevent STN and the development of additional calli, nodal explants of 21-day-old seedlings were cultured on full-strength or half-strength basal salt MSM medium supplemented with 10 µM Kin, as previously described. The medium was enriched with 0, 100 or 200 mM B (as H_3BO_3) and 2.5 or 5 mM Ca (as $CaCl_2$). The medium was dispensed into baby jars, which contained 250 ml of the medium and eight explants.

Rooting and plantlet acclimatization

Shoots longer than 3 cm were separated and cultured on half-strength MSM medium supplemented with 30 µM IAA for root induction. The plantlets were subsequently adapted to the greenhouse as described by Mohamed et al. (1999).

Statistical analyses

The experiments were arranged in a completely randomized design and performed with four replicates and repeated three times. The significance of the differences in the frequency of explants forming callus, STN, and the production of shoots and roots was calculated using Chi-square test. However, the number of developing shoots per explants was compared by analysis of variance (ANOVA) using Tukey's test at P=0.05 (Clewer and Scarisbrick, 2001) using SPSS.

Conclusion

A protocol for the in vitro micropropagation of nodal explants of the endangered desert shrub *H. persicum* was developed. The phenomenon of more severe STN symptoms was alleviated by supplementing the micropropagation medium with different concentrations of Ca and B. This protocol may permit the in vitro conservation of this multipurpose and environmentally important species.

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