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Solving the problem of shoot-tip necrosis in *Harpagophytum procumbens* by changing the cytokinin types, calcium and boron concentrations in the medium

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Abstract

Various experiments were conducted to characterize and control factors affecting shoot-tip necrosis (STN) in *Harpagophytum procumbens*. Higher cytokinin concentrations increased the incidence of STN and the problem was aggravated by the addition of auxin (IAA) to the multiplication medium. Optimum shoot multiplication was achieved by omitting auxin and using the cytokinin *meta*-Topolin riboside (*m*TR). In the presence of auxin, plantlets produced basal callus that interfered with rooting. The quantity of this basal callus was minimal when *m*TR was used. Increasing the concentration of either calcium or boron prevented the development of necrotic shoots. When the concentration of both elements was increased simultaneously, negative effects on both growth and STN were observed. Using 6 mM Ca in half-strength MS medium was optimum. Boron was toxic at higher (0.4 and 0.5 mM) concentrations. Plantlets rooted readily in half-strength cytokinin-free MS media supplemented with 2.5 μM IAA. Rooted plantlets produced, using the optimized protocol, were acclimatized successfully by direct transfer to the greenhouse in a 1:1 ratio of sand:soil mixture.

Keywords: Boron; Calcium; Cytokinins; *Harpagophytum procumbens*; Regeneration; Shoot-tip necrosis; Topolins

1. Introduction

Shoot-tip necrosis (STN) can be a major obstacle in the successful propagation of certain species by tissue culture. The symptoms of STN are browning and die back of buds and the youngest leaves. The first assumption in seeing STN is that it is caused by nutrient deficiency. The symptoms of nutrient deficiency of less mobile elements such as calcium (Ca) and boron (B) (Raven, 1977) first appear in the meristematic regions and young leaves whereas symptoms of excessive amounts of these minerals are first observed on the older leaves (Barghchi and Alderson, 1996). However, in *in vitro* systems, STN is caused by a complex set of factors rather than just nutrient deficiency. STN is a problem when culturing *Harpagophytum procumbens* with many factors such as medium composition, sugar source and frequency of sub-culturing affecting it (Jain et al., 2009).

Reports on the role of cytokinins on STN are contradictory. Some suggest that the general tendency of most protocols to omit or reduce the quantity of cytokinins to a very low level due to their anti-rooting effect causes STN (Piagnani et al., 1996). Dipping the shoot tips of apricot cultivars in benzyladenine (BA) solution, prior to transfer to rooting medium, helped overcome apical necrosis in these cultures (Perez-Tornero and Burgos, 2000) while BA had no effect on shoot tip necrosis of the pear cultivars 'William's' and 'Highland' grown *in vitro* (Grigoriadou et al., 2000). However, STN increased with higher BA

Abbreviations: BA, [6-benzylaminopurine]; MS, Murashige and Skoog medium; *m*T (*meta*-Topolin), [6-(3-hydroxybenzylamino)purine]; *m*TR (*meta*-Topolin riboside), [6-(3-hydroxybenzylamino)-9-β-D-ribofuranosylpurine]; STN, shoot-tip necrosis.

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concentration in *Quercus ruber* emphasizing that response to BA could be genotype dependent (Grigoriadou et al., 2000).

There are many reports on the role of Ca in STN both *in vitro* and *ex vitro*. These reports however lack consistency. Some report that the use of higher concentrations of Ca alleviates or controls the STN (Dyson and Digby, 1975; Vieitez et al., 1989; Singha et al., 1990; Barghchi and Alderson, 1996; Piagnani et al., 1996; Wang and Van Staden, 2001; Chang and Miller, 2005; Martin et al., 2007). In contrast, other reports show that high Ca concentrations in the culture media significantly increase the percentage of STN (Grigoriadou et al., 2000). Increasing Ca concentrations in macadamia rooting experiments failed to stop STN with concentrations higher than 6 mM aggravating the problem (Bhalla and Mulwa, 2003).

The role of B on STN is mainly due to its effect on Ca. *Ex vitro* B fertilization experiments showed some association between B and Ca in plant metabolism (Wojcik and Wojcik, 2003). In micropropagated potatoes excess B (0.1 and 0.3 mM) adversely affected Ca uptake. Media with high B levels decreased the Ca content in shoots and leaves but media with a B content of 0.025 mM, four times less than the control MS, enhanced Ca uptake (Abdulnour et al., 2000). One could therefore assume that the role of these factors on the problem of STN *in vitro* could be due to the effect they exert on one another.

Successful propagation by tissue culture of *H. procumbens* [(Burch) de Candolle ex Meissner], a widely utilized indigenous plant in southern Africa with good medicinal properties, is hampered by a high incidence of STN. Previously, factors such as composition of the medium, carbohydrate source and frequency of sub-culturing have been investigated to optimize the protocol for maximum regeneration and reducing the incidence of STN (Jain et al., 2009). Although these variables were able to partially reduce STN, the protocol could possibly be further improved by modifying other factors such as cytokinins, Ca and B levels. Thus the aim of this study was to investigate the effects of cytokinins, Ca and B on STN in *H. procumbens*.

2. Materials and methods

Previously initiated cultures of *H. procumbens* were used as the source of nodal explants. Stock cultures were sub-cultured to fresh medium every 3–4 weeks and incubated in a growth room with cool fluorescent tubes (Osram L75W/20X) at a light intensity of 45 μmol m^{-2} s^{-1} and a temperature of 25 ± 1 °C in a 16 h photoperiod. These culture conditions were used for all experiments.

The nodal explants (1 cm) were implanted into medium supplemented with 0.9% (w/v) agar and 3% (w/v) sucrose.

2.1. Effect of type and concentration of cytokinins on regeneration and STN

Meta-Topolin (*m*T) and *m*TR were synthesized in the Laboratory of Growth Regulators, Palacký University and Institute of Experimental Botany AS CR, Czech Republic. Benzyladenine was purchased from SIGMA. The effect of cytokinins on STN was assessed using half-strength Murashige and Skoog (1962) medium supplemented with 0.9% (w/v) agar and 3% (w/v) sucrose and the cytokinins BA, *m*T and *m*TR at concentrations of 5 and 10 μM each with and without the auxin IAA (2.5 μM). Each treatment had four replicates with six nodal explants each. After four weeks of growth, growth parameters such as average number of shoots per explant, percent necrotic shoots, average shoot length and mass of basal callus (separately analysed) were measured and analyzed using the SPSS package version 15.0.

2.2. Effect of Ca and B on STN

To assess the effect of Ca and B on STN, a three-step experiment was designed. Treatments in step one contained a constant B concentration (as in MS medium) and varied levels of Ca (6, 9, 12, and 15 mM), step two contained a constant Ca concentration (as in MS medium) and varied levels of B (0.2, 0.3, 0.4 and 0.5 mM) and step three had proportionally varied levels of both Ca and B (6 mM Ca+0.2 mM B, 9 mM Ca+0.3 mM B, 12 mM Ca+0.4 mM B and 15 mM Ca+0.5 mM B). The concentrations of both elements in MS medium (3 mM for Ca and 0.1 mM for B) were taken as standard concentrations. The control plants were cultured in full-strength MS medium. Other medium components were kept constant. Each treatment consisted of four replicates each with 6 explants per screw cap jar containing 40 ml medium. After four weeks, growth parameters such as average shoot length per jar (cm), fresh and dry weight (g) and average number of STN per jar were recorded and analysed using SPSS version 15.0. Mean separation by least significant difference (LSD) was done using Duncan's Multiple Range Test.

2.3. Rooting and acclimatization of tissue cultured H. procumbens

Based on the results of the above experiments, a protocol to successfully multiply the plants was established. This protocol involved the use of auxin-free half-strength MS medium with 6 mM calcium, 3% sucrose, 0.1 g/l *myo*-inositol and 5 μM *m*TR solidified with 1% (w/v) agar after adjusting the pH to 5.8. Forty ml of the medium were added to 250 ml screw-cap jars after autoclaving. Six to ten nodal explants per jar were used without notable effect on growth and multiplication. After three weeks growth under the conditions outlined previously, regenerated plantlets were transferred to cytokinin-free rooting medium containing half-strength MS media supplemented with 2.5 μM IAA and the same supplements as above. The transfer to rooting medium was preceded by careful trimming of the basal callus, when the first symptom of tissue browning was observed after three weeks. This initial attempt to root plantlets was successful and hence no additional rooting experiment was needed.

After three weeks growth in the rooting media, two sets of rooted plantlets (30 plantlets each) were potted in a 1:1 mixture of sand:soil for *ex vitro* acclimatization. The first set was transferred to the mist house and the second set transferred directly to the greenhouse. To evaluate their ability to produce tubers, fully acclimatized plants were transferred to bigger pots containing the same potting mixture and left to grow for one

year in the greenhouse with a watering frequency of once per week.

3. Results and discussion

3.1. Effect of type and concentration of cytokinins on regeneration and STN

Plants cultured in media containing BA were affected by STN at all concentrations (Fig. 1A, B). The control plants (with no plant growth regulators) showed no symptoms of necrosis but failed to grow and remained stunted (Fig. 1C). The symptom of STN started randomly in all cultures after nine days in culture. With increased duration in culture, the number of plants affected by STN increased until all plants were affected by the end of the sixth week. The severity of the problem was also associated with cytokinin (BA) concentration. At higher cytokinin concentrations, a more pronounced death that included necrosis of most leaves was observed. The death of the apical shoot resulted in lateral shoot emergence. The lateral shoot that developed first assumed the role of the apical shoot and was the next to be affected by STN, with necrosis continuing hierarchically until all shoots were affected. Plants cultured on media containing both auxin and BA and BA alone showed necrotic symptoms although the problem was more severe when auxin was added to the media. Results also showed that STN is affected by the auxin:cytokinin ratio with a higher ratio of auxin having a more pronounced effect. Treatments containing *m*TR had lower percentages of necrotic shoot-tips when compared with BA and *m*T (Table 1).

All the cytokinins tested, with or without auxin, totally inhibited rooting. Plantlets also accumulated a mass of callus-like tissue at their base (Fig. 1A, D). The amount of this callus-like tissue varied between the treatments increasing with an increase in cytokinin concentration and the addition of IAA to the medium (Fig. 2). This basal callus totally inhibited rooting, and it was an absolute requirement to trim off all basal callus before transferring to the rooting medium for rooting to occur. Vieitez et al. (1989) observed the development of basal callus on vigorously growing chestnut and oak cultures and suggested that this basal callus may operate as a sink that traps some medium components which otherwise would have helped the plant to overcome certain deficiency symptoms. The association of callus induction with the accumulation of Ca^{2+} was reported for melon cultivars. This suggested that the development of basal callus could be due to Ca^{2+} accumulation at the base of the plantlet, a phenomenon which may result in Ca deficiency in the upper part of the plantlets (Kintzios et al., 2004). Cytokinins play a role in the regulation of apical dominance and the transmission of nutritional signals (Sakakibara, 2004; Ongaro and Leyser, 2008)). Any irregularity in the action and availability of cytokinins is, therefore, most likely to cause disruption in the nutrient balance of the whole plant.

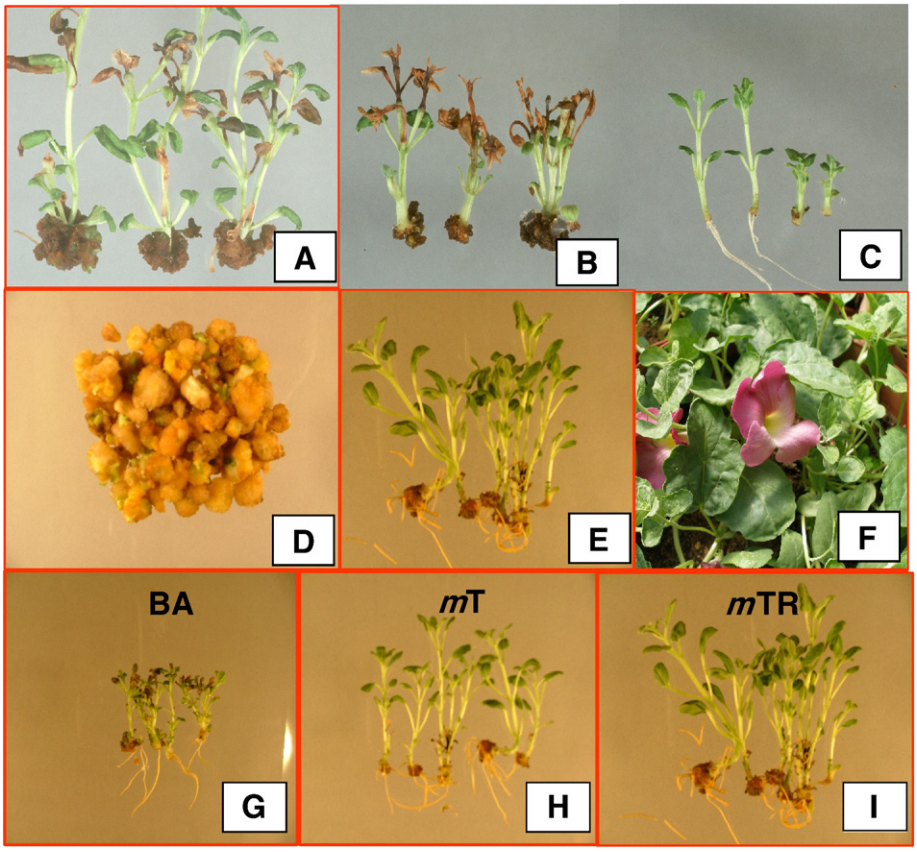

Fig. 1. *In vitro* grown *H. procumbens* (A) necrotic symptoms and development of basal callus on four-week-old culture; (B) example of STN at its worst (BA-treated); (C) two-month-old plantlets cultured in hormone free medium; (D) trimmed basal callus; (E) rooted plants ready for acclimatization (*m*TR-treated); (F) fully acclimatized and greenhouse-grown plants and (G-I) rooted BA, *m*T and *m*TR-treated respectively.

Table 1
Effect of *m*T and *m*TR, with and without IAA, on growth and shoot-tip necrosis of *H. procumbens*

Treatment (μM)	Shoot length (cm)	No. of branches	NNoST	NNeST	Fresh weight (g)	Necrotic shoots (%)
Control	3.03±0.2^{ab}	1.5±0.1^{c}	1.5±0.2^{bc}	0 ^{c}	0.14±0.02^{cd}	0
5 BA	4.81±0.3^{a}	4.3±0.4^{b}	2.9±0.4^{ab}	1.4±0.3^{bc}	1.69±0.32^{ab}	32
5 *m*T	4.05±0.1^{ab}	5.9±0.8^{a}	3.8±0.6^{a}	2.1±0.4^{b}	0.99±0.22^{bc}	35
5 *m*TR	4.00±0.2^{ab}	4.4±0.4^{b}	3.3±0.3^{a}	1.1±0.2^{bc}	0.56±0.07^{c}	25
5 BA+IAA	3.43±0.1^{ab}	6.2±0.6^{a}	2.4±0.6^{ab}	3.8±0.5^{a}	2.26±0.13^{a}	61
5 *m*T+IAA	3.75±0.1^{ab}	6.9±0.4^{a}	2.6±0.5^{ab}	4.3±0.5^{a}	1.39±0.15^{b}	62
5 *m*TR+IAA	3.60±0.4^{ab}	5.3±0.4^{ab}	3.3±0.5^{a}	2±0.3^{b}	1.31±0.09^{b}	37
10 BA+IAA	6.78±2.9^{a}	4.2±0.4^{b}	2.1±0.3^{ab}	2.1±0.4^{b}	2.03±0.18^{a}	50
10 *m*T+IAA	3.35±0.1^{ab}	7.2±0.5^{a}	4.3±0.3^{a}	2.9±0.7^{ab}	2.1±0.19^{a}	40
10 *m*TR+IAA	2.90±0.2^{ab}	6.9±0.4^{a}	4.6±0.5^{a}	2.3±0.3^{b}	2.45±0.36^{a}	33

NNoST=number of normal shoot-tips; NNeST=number of necrotic shoot-tips. Results are shown as mean±SE. Similar letters in the same column indicate no significant differences between the means.

Another way of explaining the superior effects of the topolins (*m*T and *m*TR) is their less toxic effect and easily degradable metabolites as opposed to BA. The main metabolite of BA, [9G] BA, is more stable but had a negative impact on rooting and acclimatization on cultures of *Spathiphyllum floribundum* when compared with the main metabolite of *m*T, the O-glucoside, which was degraded easily during acclimatization (Werbrouck et al., 1996). In an *ex vitro* experiment during an acclimatization period of four weeks, plants treated with *m*T produced a significantly higher number and longer roots than those treated with BA (Werbrouck et al., 1996). Addition of the aromatic cytokinin *m*TR to the culture medium significantly improved survival of potato cultures (Baroja-Fernandez et al., 2002). Control of hyperhydricity was also possible using the cytokinin *m*T in *Aloe polyphylla* cultures (Bairu et al., 2007). These results may provide some explanation for the results we found in this study.

3.2. Effect of Ca and B on STN

High concentrations of Ca in the medium reduced STN without affecting growth and regeneration. Elevated B concentration was able to reduce incidence of STN but was toxic to the plant and inhibited growth considerably (Table 2). Simultaneous addition of higher levels of these ions had a detrimental effect on growth and STN. The incidence of STN was lower when these ions were added simultaneously at elevated concentration compared to lower concentrations (Table 2). The amount of Ca and B in MS medium is considered as standard concentrations. Terms like lower and higher concentration are therefore made using this as reference.

There are a number of reports indicating that increasing the concentration of Ca and/or B in the culture medium reduces the problem of STN (Vieitez et al., 1989; Kataeva et al., 1991; Barghchi and Alderson, 1996; Piagnani et al., 1996). Calcium is involved in cellular growth and differentiation, cell wall formation, enzymatic activity and membrane permeability (see Hirschi, 2004 and Hepler, 2005 for reviews) and thus Ca deficiency could result in the disturbance of metabolic activities of growing tissues; which in turn results in growth abnormality such as STN. Abdulnour et al. (2000) studied the effect of B on Ca uptake in micropropagated potatoes and found that excess B can adversely affect Ca uptake. Medium with a high B level decreased Ca content in shoots and leaves but medium with a B

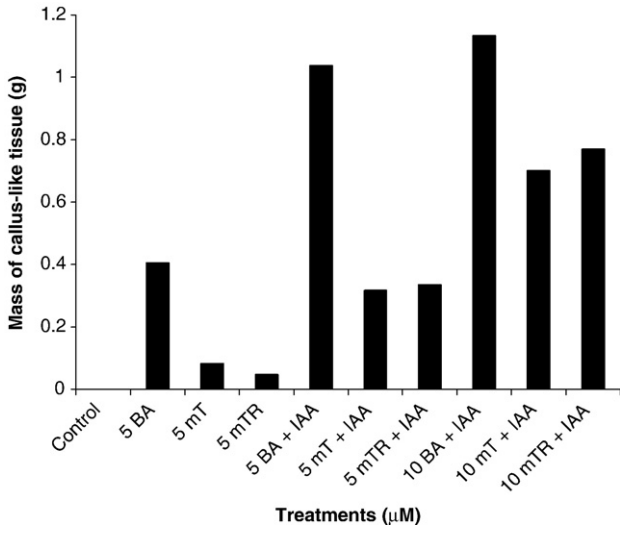

Fig. 2. The effect of BA, *m*T and *m*TR (5 and 10 μM) with or without 2.5 μM IAA on the development of basal callus.

Table 2
The effect of calcium and boron concentration on growth and shoot-tip necrosis of *H. procumbens*

Treatments (mM)	Average shoot length (cm)	Fresh weight (g)	Dry weight (g)	Mean No. of necrotic shoots per jar
Control*	5.26±0.2^{a}	6.98±0.2^{ab}	0.23±0.02^{ab}	4.8±2.6^{b}
6 Ca	4.36±0.3^{ab}	6.35±0.7^{abc}	0.23±0.01^{ab}	1.6±0.6^{bcde}
9 Ca	4.97±0.2^{a}	5.89±1.3^{abc}	0.20±0.06^{ab}	3.2±1.3^{bcde}
12 Ca	4.27±0.3^{ab}	7.57±1.1^{a}	0.33±0.06^{a}	1.4±0.9^{bcde}
15 Ca	4.70±0.3^{ab}	5.98±1.2^{abc}	0.18±0.05^{ab}	1.0±0.6^{cde}
0.2 B	4.58±0.4^{ab}	7.43±0.8^{a}	.032±0.03^{a}	4.4±0.9^{bc}
0.3 B	4.37±0.2^{ab}	7.16±1.4^{ab}	0.30±0.06^{ab}	3.2±0.9^{bcde}
0.4 B	4.08±0.5^{ab}	4.81±1.2^{abc}	0.24±0.06^{ab}	0.4±0.4^{de}
0.5 B	3.68±0.36^{b}	3.69±0.84^{c}	0.16±0.03^{b}	0.0±0.00^{e}
6 Ca+0.2 B	4.47±0.28^{ab}	4.81±1.21^{abc}	0.25±0.04^{ab}	8.0±1.14^{a}
9 Ca+0.3 B	3.68±0.45^{b}	4.04±0.64^{bc}	0.24±0.04^{ab}	4.0±1.41^{bcd}
12 Ca+0.4 B	3.50±0.28^{b}	3.46±0.67^{c}	0.22±0.03^{ab}	1.2±0.37^{bcde}
15 Ca+0.5 B	3.63±0.51^{b}	4.31±0.61^{abc}	0.24±0.03^{ab}	1.80±0.49^{bcde}

Results are shown as mean±SE. Similar letters in the same column indicate no significant differences between the means.
* The control plants were cultured in half-strength MS media.

content four times less than that of MS enhanced Ca uptake. This could therefore, provide an explanation for our results where increasing the concentration of both B and Ca had an inhibitory effect on the growth of *H. procumbens* plantlets.

Another explanation for the effect of Ca in STN would be its mode of transport in the plant system. Hirschi (2004) in his review indicated that Ca in the xylem sap needs a transpiration system for upward transport and translocation and indicated the immobile nature of this ion once deposited. The apparent lack of an efficient transpiration stream in culture vessels and in explants coupled with the limited mobility of this ion could be the causes for the deficiency symptoms observed in the form of STN in young tissues of *H. procumbens*. Shoot-tip necrosis was observed in *H. procumbens* cultures with the highest concentrations of Ca (up to 15 mM, five times more than the normal full strength MS medium), indicating that the problem of transport of this ion could have been a major contributor to STN rather than its concentration in the medium.

Increasing the concentration of B in the medium to more than 200 μM significantly reduced STN but it also reduced shoot multiplication in *Pistacia vera* (Barghchi and Alderson, 1996). The results for *H. procumbens* concur with this. Boron deficiency in vascular plants (characterized by cessation of cell division in the apical meristem) could lead to a number of secondary effects such as perturbation of auxin metabolism, increased lignification and phenol accumulation (Barghchi and Alderson, 1996). This could be the case in this study as the availability and uptake of these ions is influenced by one another.

3.3. Rooting and acclimatization of tissue cultured H. procumbens

The initial attempt to root the plantlets produced 100% rooting in just 10 days after transfer to rooting medium (Fig. 1E). An interesting observation was that when plantlets with necrotic symptoms were transferred to the rooting medium, they were capable of reverting to normal or at least, their growth and rooting *in vitro* was not affected. This observation is contrary to some reports which suggest that the possible cause of STN is the omission of cytokinins from the rooting medium to counteract their anti-rooting activity (Vieitez et al., 1989; Kataeva et al., 1991; Piagnani et al., 1996). These reports indicate that the omission, or reducing to very low level of cytokinin lead to depletion of cytokinin from the shoots resulting in shoot degeneration due to failed cell division. However, results of the present study suggest that STN in *H. procumbens* is a more complex phenomenon controlled by multiple factors. The fact that STN increased with an increase in cytokinin concentration indicated that cytokinins play a negative role.

There are consistent reports indicating that inclusion of auxin in culture media aggravates STN (Barghchi and Alderson, 1996). However, *H. procumbens* plantlets cultured on rooting media containing IAA alone did not suffer STN, suggesting that the effect of different media components on STN could be species dependent. Alternatively, the negative effect of auxin could be due to its interaction with cytokinin.

When transferring rooted *H. procumbens* plantlets to pots, it was observed that the roots were very weakly attached to the plant and easily broke off. It was therefore, imperative to carefully separate the plants from the agar medium with at least some of their roots attached. This problem led to the idea of using a liquid rooting medium. This was tried but resulted in serious hyperhydricity (results not presented).

After carefully removing *H. procumbens* plants from the rooting medium using water, plants were potted in a 1:1 ratio of sand:soil. All of the plants kept in the mist house were dead within one week after transfer. Plants transferred directly to the greenhouse, with a daily light watering for the first week, once every three days for the second week and a watering frequency of once a week thereafter had a survival rate of more than 70%. After six weeks growth, acclimatized plants were re-potted to bigger pots for tuber formation. These plants became well established, grew vigorously and produced flowers and tubers (Fig. 1F).

3.4. Aeration and rooting effects

Throughout this study it was observed that *H. procumbens* cultures in culture tubes sealed with Parafilm were more prone to STN, excessive branching (reduced elongation) and hyperhydricity as opposed to those in loosely closed screw-cap jars where plants were much healthier (results not presented). Similar results were reported for 'Norland' shoot cultures (*Solanum* spp) with plants grown in Parafilm-sealed vessels having reduced weight and increased branching. This was particularly evident in cultures with low Ca levels (Sha et al., 1985). Aeration plays a considerable role in plant growth and development via its effect on the transpiration stream. The transpiration stream plays a pivotal role in the translocation of mineral nutrients through the xylem. A high moisture vapour loss from culture vessels containing *Dianthus* microplants increased microplant establishment and calcium accumulation in the leaves and enhanced stomatal functioning, presumably by increasing transpiration *in vitro* (Cassells and Walsh, 1994). Therefore, increased rates of transpiration would most likely result in an increased rate of nutrient flow to the actively growing meristematic regions (Biddulph et al., 1961; Kohl and Oertli, 1961; Bowen, 1972; Barghchi and Alderson, 1996) and a reduced transpiration stream, reduced nutrient flow to growing shoots, resulting in the deficiency of some mineral nutrients. This problem is particularly serious in tissue culture systems where culture vessels are characterized by a very humid atmosphere which suppresses the transpiration stream of cultured shoots. In such cases other mechanisms such as root pressure become limited, reducing the ability of plantlets to absorb nutrients from the culture medium. In the present study, rooted plantlets of *H. procumbens* treated with *m*T and *m*TR slowly overcome the problem of STN when transferred to the rooting medium. BA-treated plants failed to show a similar recovery (Fig. 1G-I). Similar recovery from STN has been reported in rooted shoots of *Pistacia vera* L. (Barghchi and Alderson, 1985) and jacket plum (Mng'omba et al., 2007).

Our findings suggest that plants in a multiplication stage having no or only few roots could suffer from mineral deficiency due to a reduced transpiration stream and absence of

root pressure. The problem becomes particularly serious in the case of less mobile minerals such as Ca and B (Raven, 1977) where the demand by the plantlets exceeds the supply from the medium. Although this problem can partly be alleviated by improving aeration in the culture vessels as was noted in *H. procumbens* cultures, it should be done carefully as excessive reduction of vessel humidity can result in reduction in, or total inhibition of, shoot multiplication due to tissue desiccation caused by increased evaporation. Excessive aeration could also compromise the sterility of the culture environment by giving access to pathogens (Sha et al., 1985).

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