Thidiazuron (TDZ) induced organogenesis and clonal fidelity studies in *Haloxylon persicum* (Bunge ex Boiss & Buhse): an endangered desert tree species

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Abstract *Haloxylon persicum* (Bunge ex Boiss & Buhse), is one of the hardy woody desert shrubs, which is now endangered and/or nearing extinction. Urban landscape development and overgrazing are the major threats for the erosion of this important plant species. For conserving the species, it is critical to develop an efficient in vitro regeneration protocol for rapid multiplication of large number of regenerants. Leaf explants, cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of thidiazuron (TDZ) (0.5, 1, 2 μ M), showed significant difference in bud sprouting and

adventitious shoot induction. The highest shoot bud formation was recorded on MS medium supplemented with 0.5 μ M TDZ. Shoot tip necrosis (STN), observed after first subculture of shoot buds in same medium, increased in severity with subculture time. Application of calcium (4 mM) and boron (0.1 mM) in combination with kinetin (10 μ M) in the subculture medium significantly reduced the intensity of STN. On an average eight shoots/explant were produced by alleviating this problem. ISSR marker analysis revealed monomorphic banding pattern between progenies and parents, indicating the true to type nature of the clones and its parents.

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Introduction

Haloxylon persicum (Bunge ex Boiss & Buhse), is one of the desert woody shrubs that belong to the family of *Amaranthaceae*. It is also known as *Ghada* in Arabic. The species, originated in central Asia, is seen distributed in the Middle East, Iran, Afghanistan, North West China and near Eastern deserts (Butnik et al. 1991; Iljin 1936; Mandaville 1986; Zohary 1973). Xerophytic adaptations of the plant combat the hostility of extremely high temperature during summer months and the drought conditions of the desert ecosystem (Casati et al. 1999). It has a stout rough stem, with light grey bark, growing up to 2–4 m height. The plant has large underground root system and small branches (Ruan et al. 2006), with great potential to be used in native urban landscaping considering the ability to combat hostility of weather prevailing in the arid region and maintenance under economic use of irrigation water. It is being

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used as firewood and in the preparation of commercial charcoal. Unscrupulous grazing of plants, extensive use as firewood and fodder and climate change are the major reasons projected for the erosion of the species in deserts. Poor seed germination rate and lack of viability are also major threats for the endangerment of the plant (Birnbaum et al. 2000).

In vitro regeneration is one of the promising techniques for the mass production of genetically uniform, and pathogen-free plantlets, which can be acclimatized in short period of time (Rathore et al. 2005). The in vitro regeneration of *Haloxylon aphyllum* was reported by Birnbaum (2001) on full-strength MS medium containing 2.5 mg/l BA, using cotyledonary nodal explants. Al-Khalifah (2004) reported the micropropagation of *H. persicum* using mature seeds and isolated embryos. Low organogenesis rate and reduced shoot elongation were reported as the major problems encountered in in vitro culture of *H. persicum*. Mohamed et al (2013) reported the production of an average of 4.5 shoots of the *H. persicum* using different concentrations of BA and Kin under in vitro conditions. Thidiazuron (TDZ), a substituted phenyl-urea was recognized as one of the highly active plant growth regulators in the class of cytokinins which can induce plant morphogenesis (Murthy et al. 1998). Nevertheless, recent researches confirmed the dual role of TDZ in inducing the various morphogenic responses and other functions that auxin and cytokinin exhibit (Jones et al. 2007). TDZ induced successful plant regeneration was reported in many plant species (Guo et al. 2011).

Shoot-tip necrosis (STN) is one of the commonly occurring physiological disorders observed in plant tissue cultures, which results in the browning of the apical shoots and subsequent mortality of shoots (McCown and Sellmer 1987). The reason for STN was identified with different cultural conditions. The role of growth regulators, salt formulations, culture duration, use of additives, pH fluctuation, rooting difficulties were reported as some of the factors associated with STN (Vieitez et al. 1989; Kataeva et al. 1991; Lakshmi and Raghava 1993; Piagnani et al. 1996; Seling et al. 2000; Grigoriadou et al. 2000; Wang and Van Staden 2001).

Genetic uniformity of the clonal propagule is considered as one of the important prerequisites in micro-propagation of any plant species. Somaclonal variation among the progenies and parents is the major drawback encountered under in vitro culture conditions. Use of molecular markers is a promising technique for confirming the true to type nature of progenies. Among the different molecular markers used in clonal fidelity, ISSR marker is considered as very simple, cost-effective, fast, highly discriminative and consistent (Pradeep et al. 2002). The use of ISSR for clonal fidelity studies of micro-propagated plants was

reported earlier by many researchers (Venkatachalam et al. 2007; Yuan et al. 2009; Tiwari et al. 2013; Soumen et al. 2016). Hence the present study was conducted with the following objectives.

1. Mass multiplication of genetically uniform clones of *H. persicum* by direct or indirect organogenesis using different concentrations of TDZ under in vitro culture system.
2. Histological analysis to study developmental stages of the cultures and scanning electron microscopy to confirm the formation of shoot buds and stomatal status at initiation stage.
3. ISSR marker analysis to confirm the genetic uniformity between the progenies and the parents.

Materials and methods

Plant material

Two-year-old seedlings of *H. persicum*, 40–60 cm in height, provided by Environmental Agency, Abu Dhabi were used for the experiments. The seedlings were maintained under greenhouse conditions at a simulated temperature regime of 30–32 °C and 40% shade. Tender leaves collected from the apical region of the seedlings were utilized as explant for the tissue culture. The explants were washed thoroughly with running tap water for five minutes before it was treated with 10% clorax solution (5.25% sodium hypochlorite) with two drops of Tween 20 for 5 min. The explants were then washed in sterile water. Under aseptic condition, the explants were again treated with 20% clorax for 10 min and washed thoroughly with sterile water 4–5 times to remove any traces of chemicals.

Media preparation

Murashige and Skoog (MS) medium was used for the experiment. MS medium supplemented with different concentrations of thidiazuron (TDZ) (0.5, 1 and 2 µM) and hormone free MS medium as control was prepared by adding 3% sucrose. The pH of the medium was maintained at 5.8 prior to autoclaving at 121 °C for 15 min at 105 kPa. 0.8% agar was used as gelling agent.

Explant inoculation and culture conditions

Sterilized tender leaf explants were cut into small pieces of 1–1.5 cm length and inoculated in different concentrations of TDZ medium under sterile condition. Ten explants were inoculated in each plate, with three replications. All

cultures were incubated at 25 ± 2 °C under dark condition for 3 weeks.

Plant proliferation and alleviation of shoot tip necrosis

The adventitious bud developed from the explants were cut into small pieces under aseptic conditions and were inoculated on MS medium supplemented with TDZ (0.5, 1 and 2 μ M) for organogenesis. The cultures were maintained at 25 ± 2 °C under 16 h photoperiod and 8 h dark condition. The number of shoot buds and the size of the callus developed at the base of the buds were recorded after 3 weeks of sub-culturing. The observed callus was translucent non-regenerative in nature. Shoot tip necrosis was observed in all the cultures after second subculture. To control the STN, shoot buds developed from TDZ supplemented media were transferred to MS medium supplemented with different carbon sources like sucrose (60, 120 and 180 mM), glucose (110, 220 and 330 mM) fructose (110, 220 and 330 mM) and maltose (60, 120 and 180 mM). The effect of calcium and boron on shoot tip necrosis was also tested by transferring the shoot buds to MS medium supplemented with Kin (10 μ M) and two concentrations of CaCl_2 (2 and 4 mM) alone and in combination with H_3BO_3 (0.1 and 0.2 mM) for 3–4 weeks. Sub-culturing was done in 3 weeks interval for shoot induction.

Root induction and acclimatization

After 12 weeks of culture initiation, 3–5 cm long healthy shoots were selected and cultured on half strength MS medium supplemented with NAA (10 μ M) for root formation. Cultures were maintained in rooting medium for 6 weeks with sub-culture interval of 3 weeks. Acclimatization was performed as described by Kurup et al (2014).

DNA extraction and ISSR analysis

For clonal fidelity analysis, six progenies were selected from in vitro developed plantlets with roots, from the rooting medium. The DNA was extracted from the selected progenies and mother plant using genomic DNA purification kit (Promega). Leaves were collected from in vitro plantlets and the extract was prepared in mortar and pestle using liquid nitrogen. The quantity of isolated DNA was measured in Nanodrop and was analyzed in 1% agarose gel electrophoresis. Genetic uniformity of the in vitro propagated plants was tested using ISSR markers. Eight ISSR primers were used for the study. The PCR amplification was performed using master mix kit (Qiagen). PCR amplifications were carried out on a total volume of 20 μ L

reaction containing 10 μ L master mix, 2 μ L primer (0.2 μ M), 2 μ L of 25 ng template DNA and 6 μ L of DNase free water. The PCR reaction was performed in a Master cycler (Nexus Gradient-Eppendorf) with the following cycling profile: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 40 s at 94 °C, 45 s at 40 °C and 1:45 s at 72 °C with a final extension for 10 min at 72 °C.

The banding pattern of the amplified products were visualized using agarose gel electrophoresis using 1.5% agarose gel in 1X TBE buffer and ethidium bromide as staining agent. The electrophoresis was done at 100 V for 3 h and the bands were visualized under UV light using a gel documentation system (Cleaver scientific). PCR reactions were repeated twice to confirm the reproducibility of the results. DNA ladder (1 kb) was used as the molecular standard to confirm the appropriate size of ISSR markers. The ISSR bands were scored in excel sheet for detecting the variations between the progenies and its mother plant.

Histology and scanning electron microscopy analyses (SEM)

For histology and SEM analyses, protocols developed by Gnasekara et al. (2016) was applied for this study.

Statistical analysis

The experiment was repeated twice and three replications were made for each treatment. Data were analyzed by analysis of variance (ANOVA) followed by Tukey's range test for mean comparison.

Results and discussion

Adventitious bud induction and organogenesis

The leaf explants of *H. persicum* performed very well under in vitro culture conditions. The adventitious bud initiation from the leaf explants was observed in all the media combinations tested, within 1 week of culture initiation. MS medium supplemented with 2 μ M TDZ showed the highest bud induction (97.2 ± 1.8). In control (MS hormone free), 50% bud induction was observed. The percentage of adventitious bud formation increased significantly with increase in TDZ concentration. The developed adventitious buds sub-cultured on to medium with same concentrations of TDZ resulted in shoot bud induction. The initiation of shoot buds was observed within 2 weeks of culture establishment (Fig. 1). Reports suggest that TDZ was found to be better for shoot regeneration compared to BA (6-benzyladenine) (Victor et al. 1999; Gairi and Rashid 2004). In some of the cultures, callus

Fig. 1 *H. persicum* showing different stages of development under *in vitro* condition. **a** Adventitious bud formation from explant and callus formation at the base in MS medium supplemented with 2 μ M TDZ. **b** Induction and multiplication of shoot buds in 0.5 μ M TDZ media. **c** Shoot induction after 6 weeks of culturing. **d** Histology

analysis showing the formation of meristematic structures at the surface differentiated to form numerous shoot primordia. **e** Presence of tracheal neoelements. **f** Formation of meristematic centres known as pro-meristem

initiation at the base of the adventitious buds was observed after 1 month of culture initiation. Increased proliferation of callus was observed in medium supplemented with 2 μ M TDZ (95.8 ± 3.1). The increased level of TDZ higher than 1 mM induced the formation of callus, somatic embryo or adventitious shoots (Murthy et al. 1998). Huetteman and Preece (1993) reported that higher concentrations of TDZ could lead to callus formation by reducing the axillary shoot proliferation in woody plants. The size and nature of the callus also varied in different treatments (Table 1). In all the cultures, the nature of callus appeared to be translucent non-regenerative type. The formation of shoot meristem from the callus was insignificant, compared to direct organogenesis. The optimal shoot bud induction through direct organogenesis was recorded on medium supplemented with 0.5 μ M TDZ (65.1 ± 1.9)

(Table 1). Direct organogenesis was reported to be higher in TDZ supplemented medium, by inhibiting the apical meristem growth and inducing the formation of axillary and/or adventitious buds directly from the shoot tips (Lu 1993).

The histological analysis of the developing shoot buds with callus revealed the formation of meristematic structures at the surface that differentiated to form numerous shoot primordia along the surface (Fig. 1d). Figure 1e shows the presence of tracheal neoelements, confirming the development of vascularization. The presence of tracheary elements in somatic embryos at the cotyledonary stage was reported in *Tilia cordata* (Kärkönen 2000). The formation of epidermal cell layers on the surface was also observed during the course of development. The formation of

Table 1 Effect of different concentrations of TDZ (0.5, 1 and 2 μ M) on shoot bud induction in *H. persicum*

TDZ (μ M)	Adventitious bud sprouting (%)	Callusing (%)	Callus size (cm)	Number of shoot buds/explant	Shoot tip necrosis
0	50 ± 2^d	16.5 ± 1.2^d	0.7^b	4.08 ± 1^c	100 ^a
0.5	75.1 ± 1.5^c	41.6 ± 1.6^c	0.8^b	65.1 ± 1.9^a	85.68 ± 1.2^c
1	85 ± 2.2^b	70.8 ± 2.3^b	2.3^a	43 ± 1.38^b	90.2 ± 2.3^b
2	97.2 ± 1.8^a	95.8 ± 3.1^a	0.7^b	42 ± 1.213^b	100 ^a

Value represents the mean $\pm$ standard deviation of three replicates. In a column, different letters indicate statistically significant differences between the mean values ($P \leq 0.05$) (Tukey's test)

meristematic centres, known as pro-meristem, were also observed (Fig. 1d).

Shoot tip necrosis

The occurrence of Shoot tip necrosis (STN) was noticed after 5 weeks of culturing of adventitious buds in TDZ supplemented media. The formation of STN was observed 2 weeks after the first sub culturing of shoot buds (Fig. 2). The rate of STN was severe in almost all the cultures. Increase in shoot bud sprouting at the base was observed, once the tip of main shoots died, as a result of inhibition of apical dominance (Ferguson and Beveridge 2009). Prolonged use of TDZ can also results in changes in the shoot appearance as reported by many researchers (Murch et al. 2000; Parveen and Shahzad 2010). To avoid the deleterious effect of TDZ such as fasciation of shoots, cultures were transferred to MS basal medium supplemented with 10 μ M Kin as well as growth regulator free MS medium. To rectify the problems of STN, varying concentrations of different types of carbon sources (sucrose, fructose, glucose and maltose) were tested in combination with Kin. Sucrose is one of the most commonly used carbon sources in tissue culture. Due to fast metabolism of sucrose, the incidence of hypoxia and ethanol accumulation in plant tissue culture was reported earlier. In addition, it can also interfere with osmotic potential of the media (Neto and Otoni 2003). These circumstances can hinder the uptake of vital nutrients and elements. Even though we tried different types of sugars, sucrose (120 mM) was found to be the suitable component to reduce STN (Table 2). Fructose and glucose showed slight variation in necrosis percentage under different concentrations. Maltose completely inhibited the growth by increased STN percentage. Jain et al

(2009) reported that sucrose was the optimal carbon source for limiting the STN percentage in *H. procumbens*.

The effect of calcium and boron for overcoming STN was reported by many researchers (Piagnani et al. 1996; Bairu et al. 2009; Mohamed et al. 2013). Mohamed et al. (2013) reported the use of calcium (2.5–5 mM) and boron (100–200 Mm) in combination with kinetin to alleviate the STN in *H. persicum*. In the present study, minimal concentration of Ca (2–4 mM) and B (0.1–0.2 mM) was tested to avoid any toxic effect caused by higher concentrations. Calcium is one of the major components required for the plant, which plays important role in cell growth, cellular differentiation, development of cell wall, membrane permeability and enzyme activities (Hirschi 2004; Hepler 2005). Any disturbance in the Ca level can cause the metabolic changes in the plant systems which could lead to STN. In the present study, different concentrations of Ca (CaCl_2) alone and Ca in combination with B (H_3BO_3) were tested for limiting the STN percentage (Table 3). Compared with Ca supplemented media, a significant reduction in STN rate was observed in cultures treated with the combination of Ca and B (Fig. 4a). The necrosis percentage was reduced significantly to a minimum of $16 \pm 4.1\%$ when the medium was supplemented with 4 mM CaCl_2 and 0.1 mM H_3BO_3 . Eight necrosis free shoots were observed in the same medium. Medium supplemented with combinations of Ca and B resulted in similar level of activity against STN. Medium supplemented with Ca alone resulted in significantly high percentage of STN (65–75%). The percentage of sprouting buds of the explants varied significantly under different concentrations of Ca and B. The sub-culturing interval was reduced to 2–3 weeks for better response to avoid STN. The adventitious bud sprouting was maximum in 2 μ M Kin medium supplemented with 2 mM CaCl_2 and 0.1 mM H_3BO_3 and it was on par with medium

Fig. 2 *H. persicum* showing STN **a** Early stages of STN in TDZ media after first subculture. **b** Culture showing severe necrosis after second subculture

Table 2 Effect of different carbon source on shoot tip necrosis and shoot induction

Media composition					Explants with sprouting shoot buds (%)	Number of shoots formed/explant	Percentage of shoot tip necrosis
Kin (μM)	Sucrose (mM)	Fructose (mM)	Glucose (mM)	Maltose (mM)			
10	60				62.5 ± 2.5^a	4.5 ± 1.3^{abc}	95 ± 1.5^{ab}
10	120				70.5 ± 3^a	6 ± 0.9^a	84 ± 2^c
10	180				65.3 ± 1.4^a	5.2 ± 0.5^{ab}	96 ± 2.2^c
10		110			12.5 ± 3.2^{cde}	1.1 ± 0.56^c	100
10		220			22 ± 1.5^{bcd}	5 ± 1.1^{ab}	94 ± 2.1^{ab}
10		330			19 ± 2.1^{cde}	3.5 ± 1^{abc}	98 ± 1.1^a
10			110		24.5 ± 5^{bc}	2.5 ± 0.75^{abc}	100 ^a
10			220		32.5 ± 2^b	4.2 ± 1.3^{abc}	89 ± 2.8^{bc}
10			330		18.5 ± 4.5^{cde}	3.1 ± 1.2^{abc}	96 ± 1.5^a
10				60	9.5 ± 4^{de}	1.1 ± 0.8^c	100
10				120	12 ± 3^{de}	1.5 ± 0.5^{bc}	100
10				180	7 ± 2.4^e	1.1 ± 0.5^c	98 ± 1.2^a

Value represents the mean $\pm$ standard deviation of three replicates. In a column, different letters indicate statistically significant differences between the mean values ($P \leq 0.05$) (Tukey's test)

Table 3 Effect of Ca and B on shoot bud induction, shoot development and shoot tip necrosis

Media Composition			Explants with sprouting shoot buds (%)	Number of shoots shoots formed/explant	Percentage of shoot tip necrosis	Rooting percentage
Kin (μM)	CaCl ₂ (mM)	H ₃ BO ₃ (mM)				
10	2	0	75.2 ± 2.5^b	4.2 ± 1.1^b	73.5 ± 3^a	28 ± 0.6^{ab}
10	4	0	80 ± 3^{ab}	6 ± 0.8^{ab}	65 ± 2.1^a	32 ± 1.1^a
10	2	0.1	92.6 ± 4.1^a	7.1 ± 0.5^{ab}	20.6 ± 2.5^b	18 ± 0.6^b
10	4	0.1	86 ± 3^{ab}	8.3 ± 1^a	16 ± 4.1^b	20 ± 0.5^b
10	2	0.2	60.8 ± 4.1^c	5.2 ± 1.3^{ab}	22.5 ± 3.2^b	12 ± 1.1^c
10	4	0.2	55 ± 3.5^d	3.5 ± 1^b	18.5 ± 3.1^b	8 ± 0.4^c

Value represents the mean $\pm$ standard deviation of three replicates. In a column, different letters indicate statistically significant differences between the mean values ($P \leq 0.05$) (Tukey's test)

added with CaCl₂ (4 mM) alone and combination of 4 mM CaCl₂ and 0.1 mM H₃BO₃. The shoot bud induction rate and shoot formation were significantly low in medium incorporated with 2 and 4 mM CaCl₂ in combination with 0.2 mM H₃BO₃. This result indicated that increased concentration of B could be toxic to the tissue. In *Harpagophytum procumbens*, 6 mM Ca was found to be optimum for alleviating the STN and the higher level of B concentration (0.4–0.5 mM) could cause adverse effect (Bairu et al. 2009) as observed in the present study. Necrosis free shoots was relatively high in 4 mM CaCl₂ and 0.1 mM H₃BO₃, which was on par with other combinations, except in medium supplemented with 2 mM CaCl₂.

The scanning electron microscopy of the developing shoot buds was conducted to confirm the stages of

development. Elongated cone shaped meristematic tips were observed in the early stages of bud sprouting. Cells present on the top were smaller compared to cells at the basal region. Stoma accompanied with guard cells and subsidiary cells were also observed in the cultures (Fig. 3). Advina et al. (2012) reported that the presence of large number stomata in Protocorm-like bodies (PLBs) of *Dendrobium* orchid involved in increased transpiration efficiency essential for the growth of the PLBs.

Rooting percentage was found to be higher in medium supplemented with Ca alone compared to Ca and B combinations. The maximum rooting percentage was in medium added with 4 mM Ca. The root formation was observed within 6 weeks of culturing in medium supplemented with NAA 10 μM , accomplishing complete

Fig. 3 Scanning electron microscopy study of shoot bud initiation: **a** Presence of elongated cone shaped meristematic dome. **b** Presence of stoma accompanied by guard cells and subsidiary cells

Fig. 4 Regeneration of shoot buds. **a** Regenerated shoots in medium supplemented with Kin (10 μM), Ca (4 mM) and B (0.1 mM) with no shoot-tip necrosis, **b** Elongated shoots under rooting medium, NAA (10 μM), **c** Plants under greenhouse condition

Table 4 List of primers, their sequence, annealing temperature and number of amplified products generated by ISSR analysis

Primer name	Sequences	Annealing temperature (°C)	Number of scorable bands/primer	Number of bands amplified
UBC 807	5'AGAGAGAGAGAGAGAGT3'	43	18	126
UBC 810	5'GAGAGAGAGAGAGAGAT3'	43	16	112
UBC 823	5'TCTCTCTCTCTCTCC3'	41	11	75
UBC 826	5'ACACACACACACACACC3'	41	16	112
UBC 828	5'TGTGTGTGTGTGTGA3'	41	8	56
UBC 860	5'TGTGTGTGTGTGTGAA3'	43	11	77
HB 15	5'GTGGTGGTGCC3'	40	15	105
17899A	5'CACACACACACAAG3'	41	11	77
ISSR19	5'ACACACACACACACGA3'	41	11	77
ISSR 20	5'TAGAGAGAGAGAGAGAG3'	41	7	49
			124	866

organogenesis. The rooted plantlets were transferred to Styrofoam cups containing peat moss and vermiculite in the ratio of 2:1 showing 36% survival under *ex vitro*

greenhouse conditions with RH 50–60% and a temperature range of 27 ± 2 °C (Fig. 4b, c).

ISSR analysis was performed using 10 different ISSR primers (Table 4) to observe the genetic uniformity of the

Fig. 5 DNA fingerprinting pattern of *in vitro* propagated plantlets of *Haloxylon persicum* using ISSR primer **a** UBC 826 and **b** UBC 828 Lane MP—mother plant, Lane H1–H6—micro propagated progenies, Lane M—DNA marker

progenies developed. Six healthy progenies were chosen from the *in vitro* developed plantlets along with the corresponding mother plant for the analysis. The banding patterns were scored and a total of 124 monomorphic scorable bands were detected (Fig. 5). The number of bands amplified by each primer varied from 7 to 18. All the tested primers displayed monomorphic banding pattern between the progenies and its mother plant. This monomorphic banding pattern suggested the genetic uniformity of the micro-propagated progenies and its parent. In *Moringa peregrina*, Wesam et al. (2013) reported the presence of monomorphic bands in all the progenies and parents tested for genetic uniformity. The probable genetic conformity observed between the parent and the progeny is significant in order to re-introduce the progenies at the habitat of collection since the species are designated as endangered.

Our findings suggest that application of lower concentration of TDZ in the initial stages of *in vitro* culture, showed potential impact on large-scale production of the shoot buds from a single explant of *H. persicum*. Shoot tip necrosis was found to be the major barrier associated with the clonal multiplication of *H. persicum*. Addition of Ca in combination with lower levels of B was found to be optimal for reducing the STN level. Therefore, the current protocol may have an appreciable role in the production of

genetically uniform clones of *H. persicum* under *in vitro* condition.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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