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RESEARCH ARTICLE

Insights into nuclear DNA content, hydrogen peroxide and antioxidative enzyme activities during transverse thin cell layer organogenesis and *ex vitro* acclimatization of *Malaxis wallichii*, a threatened medicinal orchid

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Abstract *Malaxis wallichii* (Lindl.) Deb, a small, perennial, monopodial, terrestrial orchid, is endemic to tropical Himalayas at an altitude of 1200–2000 m asl. The pseudobulbs are important ingredients of century old drug ‘Ashtavarga’ and a polyherbal energetic tonic ‘Chyavanprash’. An efficient genetically stable in vitro propagation protocol using transverse thin cell layer culture system was established for *M. wallichii*. In the present report, meta-topolin alone proved to be three times more beneficial compared to other routinely used cytokinins in inducing highest number of shoot buds, plant height and growth of regenerated shoots. The highest regeneration frequency (89%) along with maximum number of adventitious shoots per explant (22.5 ± 0.6) was observed in MS medium supplemented with 1.0 mg/l meta-topolin and 0.5 mg/l α-naphthalene acetic acid. Highest rooting frequency with highest number of roots (8.66 ± 0.3) was achieved in half-strength MS medium fortified with 1.0 mg/l indole acetic acid. Clonal stability of in vitro-derived plantlets was evaluated and compared to donor plant using intron splice junction (ISJ) markers and flow cytometry. ISJ markers revealed 4.76% clonal variability indicating high degree of genetic stability amongst the in vitro-derived regenerants. The nuclear DNA content of *M. wallichii* (2n) was found to be 2C = 2.760 ± 0.02 pg and therefore, 1349.64 Mbp (1C). Flow cytometry analysis of actively growing young and mature leaves from donor as well as in vitro-derived plantlets revealed presence of three peaks corresponding to 2C, 4C and 8C, while 2C was the most abundant. In the present investigation, there was no significant difference in the 2C DNA content between the mother and in vitro-derived plants; however, the frequency of endopolyploid cells varied in young and adult plants. An increased H_2O_2 content as well as lipid peroxidation activities were observed during early stages of acclimatization which declined afterwards. The enhanced activities of antioxidant enzymes like superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase in acclimatized plantlets as compared to in vitro-grown ones revealed their active involvement in growth and development against oxidative stress under external adverse environment.

Keywords *Malaxis* · Meta-topolin · Flow cytometry · Oxidative stress · Lipid peroxidation · Antioxidant enzymes

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Introduction

Orchids with more than 600–800 genera and 25,000–35,000 species, are considered to be the most diverse and highly evolved groups among angiosperms, specialized with adaptive excellence in reproductive mechanisms (Kumaria and Tandon 2007). They are highly admired in the horticultural and cut flower industries for their beauty in floral diversity, fragrance and long shelf life. Besides their ornamental values, they are used in traditional folk medicines as they are rich source of various

phytochemicals. *Malaxis wallichii* (Lindl.) Deb (syn. *M. acuminata* D. Don), commonly known as 'Jeevaka', is a small, perennial, monopodial, pseudobulbous terrestrial orchid with pale yellowish-green to pinkish flowers in terminal racemes (Fig. 1a). The herb is endemic to the tropical Himalayas on moist, shady places in the pine forests at an altitude of 1200–2200 m asl. The dried pseudobulbs are important ingredient of century old Ayurvedic drug 'Ashtavarga' (a polyherbal drug mix of eight medicinal plants) and also used in preparation of an energetic polyherbal tonic 'Chyavanprash' under different industrial brand names (Cheruvathur et al. 2010; Govindarajan et al. 2007). It is highly appreciated as immunomodulator, health promoter, rejuvenator, brain tonic and for its adaptogenic, antioxidant, cardioprotective and anti-aging properties (Balkrishna et al. 2012; Sharma et al. 2011). The plant is also known to cure tuberculosis and protect against cough and cold (Chauhan 1990; Kaur and Bhutani 2010). The species has become threatened in the nature due to rapid loss of the forest cover, jhum cultivation, over exploitation of the pseudobulb for the drug preparation and other biotic and anthropogenic interferences. *M. wallichii* has been identified as vulnerable in Western Himalayas by Conservation Assessment and Management Plan (CAMP)-WWF, India and also included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) Appendix II (Lohani et al. 2013). Explants from different plant tissues measuring very small size (0.1–5 mm) excised either transversally or longitudinally have been successfully used for shoot regeneration, somatic embryogenesis and genetic transformation (Ahn et al. 1996; Van Le et al. 1997; Nhut et al. 2000; Zhao et al. 2007 and Singh et al. 2012). Since its inception (Tranh Than Van 1973), thin cell layer (TCL) technology has been effectively used for the mass micropropagation of many angiosperms including a few orchids as TCL culture system has been reported to be more efficient than any other conventional in vitro culture method with regard to the total output of regenerants (Van Le et al. 1999; Nayak et al. 2002; Park et al. 2002; Zhao et al. 2007; Ghnaya et al. 2008; Rangsayatorn 2009; Singh et al. 2012 and Raomai et al. 2015).

Recently, there has been a growing interest to study the reactive oxygen species (ROS) and the antioxidant defense mechanisms during shoot organogenesis, embryogenesis and *ex vitro* acclimatization of in vitro-derived plants (Gupta and Datta 2003; Jahan et al. 2014). Under in vitro conditions, plants experience significant stress while exposed to extreme culture conditions (low light intensity, high relative humidity, limited gaseous exchange, higher sucrose or nitrogen levels and exogenous growth hormones) resulting in morphological and physiological disorders (Ahmed and Anis 2014a, b). The ROS production during in vitro culture has also been reported to be associated with plant recalcitrance (Benson 2000). *In vitro*-derived plantlets experienced further stress during *ex vitro* transfer to the green house or field conditions as they switch to the high light intensity, low relative humidity and higher vapour pressure between the in vitro and field conditions which can induce severe water stress, ultimately leading to the formation of ROS (Ahmed and Anis 2014a, b; Pinto et al. 2011; Varshney and Anis 2012). Plant cells have developed several antioxidant molecules (glutathione, ascorbate, α-tocopherol etc.) and enzyme systems (SOD, CAT, APX, GR, POX etc.) to protect themselves against oxidative damage by free radicals (Asada 2006).

M. wallichii is a very slow growing orchid species in nature which propagates by means of underground rhizomes, and seed germination in nature is very low (Deb and Arenmongla 2014). Though during the last few years, efforts have been made considerably on in vitro multiplication of *M. wallichii* using different explants (internodal and pseudobulb segments) and mode of regeneration, but reports on genetic stability, nuclear genome size and antioxidant enzyme activities during morphogenesis and acclimatization are still unexplored (Arenmongla and Deb 2012; Cheruvathur et al. 2010; Kaur and Bhutani 2010). In view of the medicinal and conservation importance of *M. wallichii*, the present study was designed to (1) optimize the mass multiplication of *M. wallichii* through transverse thin cell layer (tTCL), (2) assess the clonal fidelity of tTCL-derived plants by flow cytometry and intron splice junction markers (ISJ), and (3) to assess the changes in the various antioxidant enzyme activities, hydrogen peroxide (H_2O_2) content and lipid peroxidation during in vitro culture conditions and acclimatization periods.

Materials and methods

Explant source and culture conditions

The plants of *M. wallichii* with inflorescences were collected from Upper Shillong (Meghalaya) and maintained in the greenhouse of Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University (NEHU), Shillong, India. About 8–9 months old purplish-green capsules of *M. wallichii* were harvested and washed thoroughly under running tap water for 45 min. Capsules were then treated with 0.5% (v/v) Cetrimide solution containing 1% (v/v) Tween-20 for 10 min followed by thorough washing three times with sterile distilled water. Finally, the capsules were dipped in 70% (v/v) ethanol for 30 s followed by flaming for 2–3 s. The sterilized capsules were excised longitudinally with a sterile surgical blade and around 200–300 seeds were inoculated per test tube on

Fig. 1 In vitro propagation of *M. wallichii* through transverse thin cell layer. **a** Plant with inflorescence growing in the green house of Plant Biotechnology Laboratory, NEHU, Shillong, India, **b** A small green bud-like structure emerged from the brim of the tTCL explant in MS + 1.0 mg/l mT, Bar = 5 mm, **c**, **d** Neoformation of adventitious shoot buds from the base of first shoot as well as from the surface of tTCL explants in MS + 1.0 mg/l mT, Bar = 5 mm, **e**, **f** Development of multiple shoots in MS + 1.0 mg/l mT + 0.5 mg/l NAA, Bar = 1 cm, **g** Single bud-like structure developed from the tTCL explants after two weeks of culture in MS + 1.0 mg/l BAP, Bar = 5 mm, **h** Multiple shoot induction in MS + 1.0 BAP, Bar = 1 cm, **i** Effect of mT on plant morphology in vitro compared to BAP in equimolar concentration (1.0 mg/l) in MS medium, Bar = 1 cm, **j** Initiation of rooting in MS + 1.0 mg/l mT + 0.5 mg/l NAA, Bar = 1 cm, **k** Rooting of *M. wallichii* in half strength MS + 1.0 mg/l IAA, Bar = 1 cm, **l** Acclimatized plants in green house after 60 days

Murashige and Skoog (MS) medium (1962) without any plant growth regulators (PGRs). Pseudostem segments with one or two nodes (2.0–4.0 cm) cut from the in vitro-raised seedlings were used as explant sources.

Transverse thin cell layer (tTCL) culture and shoot organogenesis

Pseudostem segments with nodes were transversely cut (0.3–0.5 mm thickness) from base to shoot tip. tTCL explants were cultured for shoot regeneration on MS medium supplemented with various cytokinins, including 6-benzylaminopurine (6-BAP), kinetin (KN), N6–[2-isopentenyl] adenine (2-iP), thidiazuron (TDZ) and meta-Topolin (mT) at different concentrations (0.5–2.0 mg/l) either singly or in combination with 0.5 mg/l α-naphthaleneacetic acid (NAA). The pH of the medium was adjusted to 5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media containing culture vessels were autoclaved at 104 kPa and 121 °C for 20 min. The cultures were maintained at 25 ± 2 °C, 75–80% relative humidity and 14 h photoperiod of 50 µMol m^{-2} s^{-1} irradiance provided by cool-white fluorescence tubes (Phillips, India). The experiments were repeated three times with ten replicates per treatment.

Rooting, acclimatization and field transfer

Regenerated shoots with two to three expanded leaves grown in optimal regeneration medium were transferred to half-strength MS medium supplemented with different concentrations of indole-3 acetic acid (IAA) or NAA (0.5–2.0 mg/l). The frequency of rooting, average number of roots per shoot and average root length were measured after four weeks of culture. Regenerated plantlets with well developed roots were removed from culture vessels, and washed thoroughly with distilled water to remove any traces of solidifying agents. The regenerated plantlets were then planted in thermocol pots containing vermiculite. Plantlets were acclimatized for 4 weeks at 25 ± 2 °C under 14 h photoperiod (light 50 μMol m^{-2} s^{-1}; 75–85% relative humidity) and irrigated with 1/10th liquid MS medium without sucrose and finally transferred to greenhouse (light 70 μMol m^{-2} s^{-1}; temperature 17–25 °C and 75–85% relative humidity). Survival rate was recorded after 12 weeks of acclimatization.

Analysis of genetic stability of regenerated plantlets

Genomic DNA extraction and PCR amplification

Total genomic DNA was isolated from fresh young leaves of mother plant as well as randomly selected fifteen in vitro-raised plantlets following the method described by Porebski et al. (1997) with minor modifications. Quality and quantity of the isolated DNA was checked by using a nanodrop (Nanodrop 2000-C, Thermo Scientific, USA). After preliminary screening of 30 ISJ primers, 15 primers were selected to analyze genetic stability for their reproducible and unambiguous banding patterns. All the PCR reactions contained 30 ng of template DNA, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPss, 1 U Taq Polymerase (Merck, India) and 15 pmol ISJ primer in each of the 25 μl reaction volume. (Thermo Scientific, USA). The reaction program for ISJ was set at 94 °C for 4 min followed by 40 cycles of 94 °C for 1 min, 48–58 °C for 1 min and 2 min at 72 °C with a final extension at 72 °C for 7 min in a Veritti Thermal Cycler (Applied Biosystems, USA). Amplification with each primer was repeated twice to verify the reproducibility of the findings. All the amplified PCR fragments were resolved in 1.8% agarose gel at 80–85 V for 2.5 h in a 1× TAE buffer, stained with ethidium bromide. Amplified fragments were visualized under UV light and photographed using the Gel Documentation System (Biostep DH-20, Germany). Amplified fragments generated by ISJ primers were scored as binary data matrix based on the presense (1) or absence (0) of selected bands. Genetic similarity based on Jacard's coefficient was calculated using NTSYS pc version 2.20 (Rohlf and Taxonomy 1998) following Unweighted Pair Group Method with Arithmetic Mean (UPGMA) option of SHAN module.

Flow cytometry (FCM) analysis

The youngest fully developed leaves of donor as well as in vitro-derived plantlets were used for the preparation of intact nuclei suspension following the protocol developed by Krishan (1975) with some minor modifications for FCM analysis. Seeds and the corresponding genome size of internal reference standard [*Pisum sativum* Citrad (2C = 9.09 pg)] were kindly provided by Jaroslav Doležel, Institute of Experimental Botany, Academy of Sciences of the Czech Republic. Approximately 100 mg of selected plant tissue (1 cm^2) was co-chopped with a sharp razor blade in a plastic Petridish with 2 ml of hypotonic-propidium iodide (HPI) isolation buffer containing 0.3% (w/v) citric acid monohydrate (Sigma-Aldrich, India), 0.05% (v/v) IGEPAL CA-630 (Sigma-Aldrich, USA), 50 μg/ml propidium iodide (PI) (Sigma-Aldrich, India) and 2 mg/ml of RNase A (Himedia, India). One percent (w/v) PVP-40 (Himedia, India) and 1% (v/v) β-mercaptoethanol (Himedia, India) were occasionally added to the HPI buffer to minimize the negative effects of cytosolic inhibitors on propidium iodide fluorescence. The nuclei suspension was then filtered through a 40 μm nylon mesh filter (BD Falcon, USA) and nuclear DNA content was estimated within 20 min with a BD FACS Calibur flow cytometer (Becton–Dickinson, NJ, USA) equipped with a 15 mW 488 nm air-cooled Argon ion laser. For each sample at least 10,000 nuclei were analyzed and the results were acquired using BD Cell Quest Pro software version 6.0 (BD Biosciences, USA). The mean position of the sample peaks relative to the internal standard was compared by Frequency versus FL histogram. The analysis was performed with five replicates on three different days.

Assay of antioxidant enzymes

Extract preparation

Briefly, 0.5 g fresh leaf tissues of in vitro grown shoots as well as in vitro-raised plantlets (2 and 4 week old) were homogenized in 2.0 ml 0.5 M phosphate buffer (pH 7.5) containing 1% PVP (w/v), 1% IGEPAL (v/v) and 0.1 g EDTA using a pre-chilled mortar and pestle. The homogenates were centrifuged at 15,000 rpm for 25 min at 4 °C and the resulting supernatants were used for enzyme assays.

Superoxide dismutase (SOD)

SOD (EC: 1.15.1.1) activity measured by monitoring the inhibition of photochemical reduction of nitroblue

tetrazolium (NBT) as described by Dhindsa et al. (1981) in a reaction mixture containing 0.5 M sodium phosphate buffer (pH 7.5), 13 mM methionine, 63 mM NBT, 1.3 mM riboflavin and 0.1 ml of enzyme extract. The reaction mixture was incubated for 15 min under 15 W fluorescent lamp and absorbance was measured at 560 nm against non-irradiated blank.

Catalase (CAT)

CAT (EC: 1.11.1.16) activity was determined following the protocol described by Aebi (1984) in a reaction mixture containing 50 mM sodium phosphate buffer (pH 7.0) and 100 µl of enzyme extract in a total volume of 3 ml and the reaction was initiated by the addition of 10 mM Hydrogen peroxide (H_2O_2). The rate of H_2O_2 decomposition measured at 240 nm indicated catalase activity.

Glutathione reductase (GR)

GR (EC: 1.6.4.2) activity of extract was measured through glutathione-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) at 340 nm following the protocol developed by Ahmed and Anis (2014a). The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.5), 1.0 mM EDTA, 0.2 mM NADPH and glutathione disulphide. Finally, 0.1 ml enzyme extract was added to start the reaction and incubated for 5 min at 25 °C.

Ascorbate peroxidise (APX)

APX (EC: 1.11.1.11) activity was estimated following the protocol described by Nakano and Asada (1981) by monitoring the decreasing trend of absorbance of ascorbate at 290 nm within the 1 min duration. The assay mixture contained 50 mM sodium phosphate buffer (pH 7.5), 0.5 mM ascorbate, 0.1 mM H_2O_2, 0.1 mM EDTA and 0.1 ml enzyme extract.

Detection of H_2O_2 and lipid peroxidation during organogenesis and acclimatization

Detection of total H_2O_2 content of in vitro grown shoots as well as in vitro-raised plantlets was determined by titanium oxidation method as described by Patterson et al. (1984). The absorbance was measured at 410 nm and H_2O_2 concentration was obtained by the standard curve. Lipid peroxidation activity during in vitro organogenesis and acclimatization was estimated by measuring the level of malondialdehyde (MDA) production thiobarbituric acid (TBA) method previously described by Chakrabarty and Datta. (2008) and absorbance was measured at 534 nm.

Data analysis

All experiments were carried out with ten replicates and were repeated three times. Data were analyzed using one-way analysis of variance (ANOVA) at 0.05 significance level using JMP® version 7.0.1 (SAS Institute, Cary, NC, USA). The significant differences among the means were assayed by Tukey's Honestly Significant Difference (HSD) test on significant findings.

Results and discussion

tTCL mediated organogenesis

Transversely sliced pseudo-stem segments (0.3–0.5 mm) from the in vitro raised seedlings were cultured on MS medium fortified with BAP, KN, 2-iP, mT, TDZ (0.5–2.0 mg/l) and NAA (0.5 mg/l) either alone or in combinations. A small, green bud-like structure gradually appeared on the brim of the tTCL explants within a fortnight of inoculation (Fig. 1b) and subsequently first adventitious shoot evolved from that protruding region. On the other hand, tTLC explants cultured on basal MS medium without any plant growth regulators remained green up to 1–2 weeks, showing no morphogenic response, gradually turned brown and died. Neoformation of many adventitious shoot buds occurred directly from the surface of the explants as well as from the basal region of the first shoot and finally adventitious buds and regenerated shoots together developed a clamp (Fig. 1c–e). Moreover, the efficiency of adventitious shoot bud induction was significantly affected by the position and orientation of the tTCL explants. Shoot buds were induced more frequently if the explants were cultured in an upright orientation than the inverted orientation. The cut surface of the explants with dedifferentiated vascular cells gave rise to a number of meristematic regions followed by the formation of a protuberance on the brim of each explant which gradually differentiated into a shoot apical meristem-like structure. This neoformed shoot apical meristem-like structure further developed into an intact shoot with leaf primordium. Similar observations were reported in *Dendrobium candidum* Wall. ex Lindl. (Zhao et al. 2007), *Eucalyptus gunnii* Hook. f. (Hervé et al. 2001), *E. grandis* W. Hill. (Ito et al. 1996), *Populus* spp. (Douglas 1984) and *Rhynchostylis gigantean* (Lindl.) Ridl. (Van Le et al. 1999). Amongst the tested PGRs, mT (1.0 mg/l) alone proved to be beneficial compared to other routinely used cytokinins in inducing highest number of adventitious shoot buds per tTCL explants (16.5 ± 0.4) with 85% regeneration frequency (Table 1). The adventitious shoot bud induction frequency varied significantly with the concentration, type

Table 1 Influence of different plant growth regulators on shoot regeneration from tTCL explants of *M. wallichii*

Plant growth regulators (mg/l)						Regeneration frequency (%)	No. of shoot buds/explant	Length of shoots (cm)
BAP	KN	2-iP	mT	TDZ	NAA			
0	0	0	0	0	0	0	0	0
0.5	–	–	–	–	–	52.0	2.0 ± 0.1^{y}	3.04 ± 0.1^{s}
1.0	–	–	–	–	–	64.0	4.5 ± 0.2^{t}	4.0 ± 0.2^{o}
1.5	–	–	–	–	–	68.0	2.9 ± 0.1^{v}	3.14 ± 0.2^{r}
2.0	–	–	–	–	–	50.0	2.4 ± 0.1^{x}	2.36 ± 0.1^{t}
–	0.5	–	–	–	–	61.0	2.4 ± 0.3^{x}	3.20 ± 0.3^{r}
–	1.0	–	–	–	–	79.0	4.8 ± 0.2^{s}	4.54 ± 0.5^{n}
–	1.5	–	–	–	–	72.0	3.2 ± 0.2^{u}	4.01 ± 0.4^{o}
–	2.0	–	–	–	–	60.0	2.6 ± 0.1^{wx}	3.40 ± 0.2^{q}
–	–	0.5	–	–	–	63.0	4.4 ± 0.4^{t}	4.03 ± 0.4^{o}
–	–	1.0	–	–	–	76.0	6.8 ± 0.3^{p}	5.16 ± 0.2^{k}
–	–	1.5	–	–	–	69.0	6.02 ± 0.2^{q}	4.76 ± 0.3^{l}
–	–	2.0	–	–	–	59.0	5.2 ± 0.1^{r}	3.78 ± 0.3^{p}
–	–	–	0.5	–	–	74.0	13.0 ± 0.4^{ij}	6.26 ± 0.4^{g}
–	–	–	1.0	–	–	85.0	16.5 ± 0.4^{e}	7.15 ± 0.2^{d}
–	–	–	1.5	–	–	82.0	13.8 ± 0.6^{h}	6.86 ± 0.4^{f}
–	–	–	2.0	–	–	72.5	11.0 ± 0.3^{m}	6.14 ± 0.5^{h}
–	–	–	–	0.5	–	64.0	11.4 ± 0.3^{l}	6.1 ± 0.5^{h}
–	–	–	–	1.0	–	74.0	13.2 ± 0.4^{i}	6.86 ± 0.6^{f}
–	–	–	–	1.5	–	60.0	10.4 ± 0.2^{n}	5.8 ± 0.4^{i}
–	–	–	–	2.0	–	52.0	7.6 ± 0.1^{o}	4.6 ± 0.3^{m}
–	–	–	0.5	–	0.5	78.0	19.6 ± 0.3^{c}	7.20 ± 0.2^{d}
–	–	–	1.0	–	0.5	89.0	22.5 ± 0.6^{a}	8.40 ± 0.4^{a}
–	–	–	1.5	–	0.5	83.0	20.4 ± 0.4^{b}	7.80 ± 0.5^{c}
–	–	–	2.0	–	0.5	72.0	18.7 ± 0.3^{d}	7.01 ± 0.6^{e}
–	–	–	–	0.5	0.5	76.0	16.0 ± 0.4^{f}	7.04 ± 0.3^{e}
–	–	–	–	1.0	0.5	81.0	19.5 ± 0.5^{c}	8.01 ± 0.4^{b}
–	–	–	–	1.5	0.5	72.0	15.4 ± 0.6^{g}	6.86 ± 0.4^{f}
–	–	–	–	2.0	0.5	61.0	11.7 ± 0.3^{k}	5.56 ± 0.3^{j}

* Values represent mean ± SE of 10 replicates per treatment and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey–Kramer HSD ($P \leq 0.05$)

and combination of plant growth regulators used. A higher rate of regeneration frequency of shoot buds was observed when explants were cultured on cytokinin and auxin (0.5 mg/l NAA) containing medium. The highest regeneration frequency (89%) along with maximum number of adventitious shoots per explant (22.5 ± 0.6) was observed in MS medium fortified with 1.0 mg/l mT and 0.5 mg/l NAA (Table 1, Fig. 1f). In this study, mT not only positively influenced the regeneration frequency but was nearly thrice more effective than other conventional cytokinins (BAP, KN, 2-iP) in improving plant height and growth of regenerated shoots (Fig. 1i–j). The structural advantages of mT (hydroxylated side chain at meta position) increases the chances of formation of easily degradable end product, o-glucoside which is considered as stable cytokinin storage forms that can be converted to active cytokinin bases when required (Bairu et al. 2009; Werbrouck et al. 1996). Furthermore, its faster translocation rate and reversible sequestration of the o-glucoside at a physiologically active level over an extended period of time in plant tissue resulting in much higher multiplication rate justifies its ingenuity over other purine based cytokinins in plant tissue culture (Amoo et al. 2014; Valero-Aracama et al. 2010). The superiority of mT and its derivatives in improving shoot proliferation over the conventional cytokinins like BAP and Kn was demonstrated by some researchers in recent past (Amoo and Van Staden 2013; Aremu et al. 2012; Bairu et al. 2007; Baroja-Fernández et al. 2002; Bose et al. 2016; Gentile et al. 2014; Roels et al. 2005). However, a few reports on the use of mT in orchid tissue culture

indicate that this group of topolins could be a new source of aromatic cytokinins with high multiplication efficiency and improved physiological activity in micropropagated plants (Vasudevan and Van Staden 2011; Bhattacharyya et al. 2016). It was possible to produce an average of 22.5 buds or shoots from each tTCL explant within 40–60 days. Such higher frequencies were previously documented in a number of other species (Van Le et al. 1998, 2002; Zhao et al. 2007). However, the successful application of mT on adventitious organogenesis in the terrestrial orchid, *M. wallichii* is reported for the first time here.

Rooting and acclimatization

tTCL-derived microshoots (2–4 cm long) were excised individually and cultured in half-strength MS medium fortified with either IAA or IBA (0.5–2.0 mg/l) for in vitro rooting. The maximum rooting frequency (89.80%), highest number of roots (8.66 ± 0.3) with maximum root lengths (5.34 ± 0.08 cm) was achieved after 2 weeks of incubation in explants cultured in half-strength MS medium supplemented with 1.0 mg/l of IAA (Table 2, Fig. 1j–k). Similarly, IAA was successfully employed to enhance root formation in orchids and several other plant species (Zhao et al. 2007; Barpete et al. 2014; Pant and Thapa 2016). The regenerated plants with 3–5 healthy roots were successfully acclimatized in thermocol cups containing vermiculite. The highest survival percentage recorded was 84.5% using vermiculite compared to other potting mixtures used and feeding the plantlets with diluted 1/10th MS was found suitable for their growth during acclimatization. In the present study, mT treated plants showed positive influence on in vitro rooting (length and number of roots) and acclimatization (survival %) than BAP or KN treated ones. The present findings are in accordance with the observations made by other workers (Aremu et al. 2012; Bairu et al. 2007; Gentile et al. 2014; Valero-Aracama et al. 2010).

Genetic stability analysis of regenerated plants

ISJ analysis

Plant tissues grown under in vitro conditions have inherent limitation of induced genetic instability, commonly referred to as somaclonal variation. The occurrence of such variations during culture depends upon the types and concentration of PGR used, explants sources and pathway of regeneration (Chavan et al. 2014). *In vitro* tTCL-derived plantlets did not show any detectable phenotypic variations when compared to mother plant. Thus, regenerated plants were assessed for their clonal fidelity by using ISJ markers. In the present study, 15 randomly selected in vitro-derived plantlets as well as mother plant were used to evaluate the genetic stability. A total of 30 ISJ primers screened, out of which 15 primers that gave clear, unambiguous, reproducible and scorable bands were considered for further amplification. These 15 ISJ primers generated a total of 84 amplicons, out of which 4 bands were polymorphic with 4.76% of clonal variability (Table 3; Fig. 2a). The number of fragments varied from 4 to 8 with an average 5.6 bands per primer. The Jacard's similarity index ranged from 0.97 to 1.00 revealing a high degree of genetic relatedness (Fig. 2b) which is further supported by the work of other researchers (Bhattacharyya et al. 2014; Chavan et al. 2014; Ferreira et al. 2006; Kishor and Devi 2009; Roy et al. 2012). Semi-arbitrary ISJ markers system are based on highly conserved region of exon–intron boundary and crucial for post-transcriptional splicing in plants (Weining and Langridge 1991). The key advantage of semi-arbitrary markers is that they target a wide range of plant genomes containing introns within it by evading heterochromatic

Table 2 Effect of different auxins on in vitro rooting from micro-shoots of *M. wallichii* in half-strength MS medium

Auxins	Concentration (mg/l)	Rooting frequency (%)	Number of roots	Length of roots (cm)
Control	–	–	–	–
NAA	0.5	67.0	5.45 ± 0.1^{h}	3.92 ± 0.07^{g}
	1.0	79.50	7.56 ± 0.1^{c}	4.80 ± 0.07^{c}
	1.5	72.60	7.0 ± 0.3^{d}	4.24 ± 0.04^{e}
	2.0	59.0	6.34 ± 0.2^{g}	4.0 ± 0.06^{f}
IAA	0.5	72.60	6.56 ± 0.2^{f}	4.72 ± 0.04^{d}
	1.0	89.80	8.66 ± 0.3^{a}	5.34 ± 0.08^{a}
	1.5	76.14	8.01 ± 0.11^{b}	5.02 ± 0.06^{b}
	2.0	63.57	6.76 ± 0.1^{e}	4.79 ± 0.04^{c}

* Values represent mean ± SE of 10 replicates per treatment and all the experiments were repeated three times. Means followed by the same letter in the column are not significantly different as indicated by Tukey–Kramer HSD ($P \leq 0.05$)

Table 3 Data on ISJ primers used in the detection of genetic stability in micropropagated plants of *M. wallichii*

Sl no.	Primer name	Primer sequence (5′–3′)	Band characteristics			
			Total no. of bands	No. of mono-morphic bands	No. of poly-morphic bands	% of poly-morphism
1	ISJ2	ACTTACCTGAGGCGCCAC	4	4	–	–
2	ISJ4	GTCGGCGGACAGGTAAGT	6	6	–	–
3	ISJ5	CAGGGTCCCACCTGCA	5	5	–	–
4	ISJ6	ACTTACCTGAGCCAGCGA	7	6	1	14.28
5	ISJ7	TGCAGGTCAGGACCCT	6	6	–	–
6	ISJ8	GACCGCTTGCAGGTAAGT	7	6	1	14.28
7	ISJ11	TGCAGGTCAAACGTCG	5	5	–	–
8	IT1	CCGGCAGGTCAGGTAAGT	4	4	–	–
9	IT2	GCAGAGGGCCAGGTAAGT	8	7	1	12.5
10	IT6	CCTGGAGGCCAGGTAAGT	5	5	–	–
11	IT31	GAAGCCGCAGGTAAG	6	6	–	–
12	ET1	ACTTACCTGAGGCGCGAC	4	4	–	–
13	ET32	ACTTACCTGGGCACG	5	5	–	–
14	ET34	ACTTACCTGGCCGAG	6	5	1	16.66
15	ET36	ACTTACCTGGGGCTC	6	6	–	–
Total			84	80	4	4.76

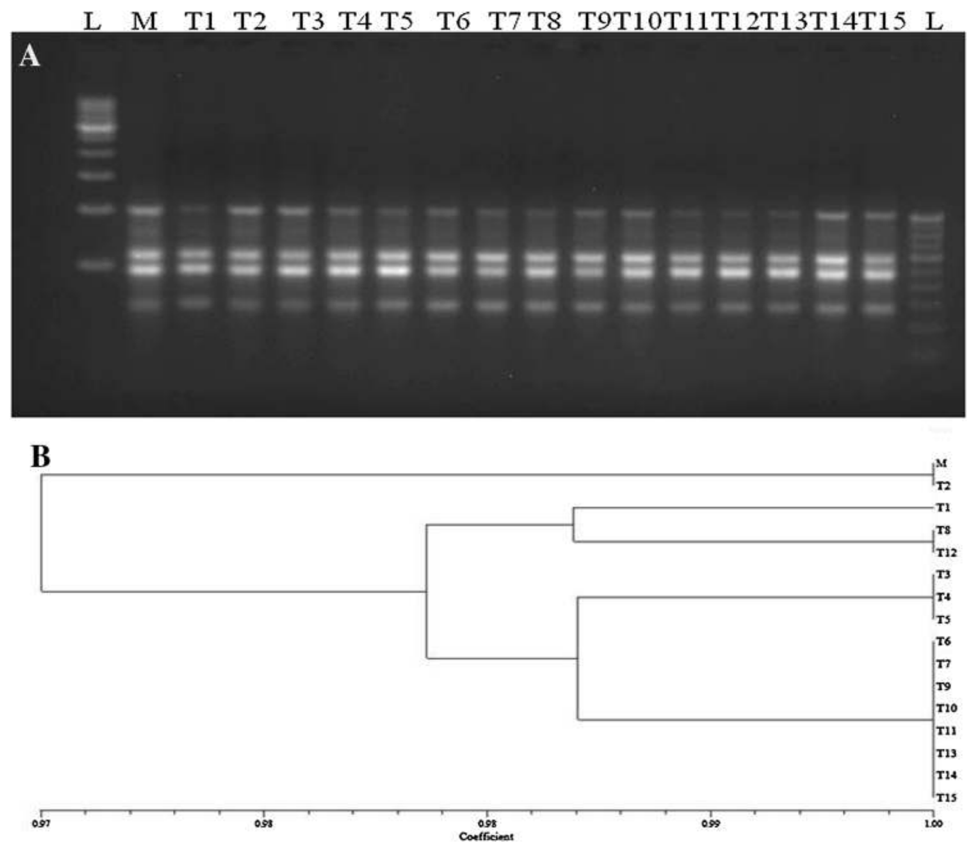

Fig. 2 Banding profile in *M. wallichii* using ISJ markers **a** IT1 primer in TMO derived plants, **b** Dendrogram illustrating coefficient similarities among regenerated plants and the mother plant by the UPGMA cluster analysis using the NTSYS-PC program; lane 1 ladder, lane 2 mother plant, lanes 3–18 in vitro-derived plants (TMO: transverse thin cell layer mediated organogenesis)

regions so as to attain a pellucid picture of genetic polymorphisms (Przetakiewicz et al. 2002). Effectiveness of ISJ markers in detecting clonal fidelity in in vitro system was previously reported by us in *Nardostachys jatamansi* (D. Don) DC. (Bose et al. 2016). In the present investigations, mT was found to be less toxic at higher equimolar concentration compared to other conventional cytokinins which could generate variations (phenotypic and genetic) during in vitro culture as supported by findings of other workers (Werbrouck et al. 1996; Bairu et al. 2007; Amoo et al. 2011; Gentile et al. 2014; Bhattacharyya et al. 2016).

FCM analysis

M. wallichii is very rich in different bioactive compounds which can interfere with the propidium iodide fluorescence during FCM analysis. Therefore, addition of 1% PVP and 1% β-mercaptoethanol to the nuclei isolation buffer proved to be beneficial in improving the quality of FCM-histograms by minimising the inhibitory effects of various cytosolic compounds produced by *M. wallichii*. The nuclear DNA content of *M. wallichii* (2n) was calculated to be 2.760 ± 0.02 pg/2C utilizing *Pisum sativum* Citrad (9.09 pg/2C) as an internal standard and therefore, 1C genome size of *M. wallichii* was estimated to be 1349.64 Mbp (1 pg = 978 bp, Doležel et al. 2007). Based on the records from Plant DNA C-values Database, *M. wallichii* genome size is approximately 8.6 times larger than *Arabidopsis thaliana* (L.) Heynh. (0.32 pg/2C) and 2.6 times that of rice (1.0 pg/2C) (Bennett and Smith 1991). Nuclei suspension isolated from actively growing young as well as mature leaves from donor and in vitro-derived plantlets revealed presence of three peaks corresponding to 2C, 4C and 8C with a cell cycle value more than 0.1 indicating endopolyploidy (Barow and Meister 2003). Young leaves from donor plants were found to have 74.56% of 2C DNA content, 17.84% of 4C and 7.60% of 8C DNA content where as mature leaves of the same showed higher 4C (28.63%) and 8C (11.03%) DNA content compared to actively growing young leaves (Table 4). tTCL mediated in vitro-derived plantlets revealed the presence of 2C, 4C and 8C nuclei with majority (78.80%) of them with 2C DNA content and about 16.0% of were 4C nuclei and the rest 8C (5.20%) (Fig. 3). A similar pattern was found in the vegetative tissues (both young and matured leaves) of one year old greenhouse-grown hardened plants. These results are in accordance with the previously published reports (Kudo and Kimura 2001; Palomino et al. 1999; Sliwinska and Lukaszewska 2005). Endoreduplication is the process of endonuclear chromosome duplication without intervening cell division and segregation, resulting in cells with multiple ploidy levels within a specific tissue or cell, designated as polysomaty or endopolyploidy (Lim and Loh 2003). Endoreduplication is widespread phenomenon in plants, particularly in angiosperms and has been documented in several species and hybrids of orchids with a very high degree of endoreduplication compared to other plant species, viz., *Dendrobium* spp., *Phalaenopsis* spp. and *Doritis pulcherrima* Lindl., *Cymbidium* spp. and hybrids, *Vanda* sp, *Oncidium varicosum* Lindl. and *Spathoglottis plicata* Blume (Fukai et al. 2002; Jones and Kuehnle 1998; Lee et al. 2004; Lim and Loh 2003; Yang and Loh 2004). In our present investigation, there is no significant difference in the 2C DNA content between the mother and in vitro-derived plants; however, the frequency of endoreduplicated cells varies in young and adult plants. To the best of our knowledge, this is the first report on genome content and ploidy information of this high value threatened terrestrial orchid.

Antioxidant enzyme assay

Under stressful environmental conditions, plant cells produce several highly reactive oxygen species such as singlet oxygen, superoxide radicals, hydrogen peroxide and hydroxyl radicals which can hamper the normal cellular metabolism through severe oxidative damage to nucleic acids, membrane lipids and proteins leading to the cell death (Jahan et al. 2014). Plants have evolved with complex antioxidant and enzymatic scavenging systems such as SOD, CAT, GR, APX, etc. to combat the extremely hazardous free radicals and thereby regulating cellular redox balance, plant growth and development (Mitrović and Bogdanović 2008). In the present investigation, continuous increase in SOD and CAT activities were observed in in vitro regenerated plantlets from second to fourth week of culture. A similar trend was observed in acclimatized plants up to second week of acclimatization period. But, unlike CAT, an abrupt decrease in SOD activity was observed by fourth week of acclimatization indicating its role in struggling to external environmental conditions (Fig. 4a, b). The decline in the SOD activity may be correlated with the reduced oxidative stress and membrane peroxidation which signifies adjustment of plant to the external stress during later phase of acclimatization period. A similar pattern of declining SOD activity during acclimatization period has been previously documented in plants such as *Tecomella undulate* Seem. (Varshney and Anis 2012), *Cardiospermum halicacabum* L. (Jahan et al. 2014), *Cassia alata* L. (Ahmed and Anis 2014a) and *Vitex trifolia* L. (Ahmed and Anis 2014b). Ubiquitous SOD is considered as a first line of defence against oxidative stress, catalyzes the dismutation of superoxide radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2) by reduction (Ahmed and Anis 2014a, b). The significant increase in CAT activity during entire in vitro culture conditions and acclimatization

Table 4 Flow cytometric (FCM) determination of nuclear DNA content in *M. wallichii*

Regeneration pathway/tissue organ	*Pisum sativum* Citrad (internal reference standard) − 2C = 9.09 pg						
	2C DNA content (pg)	1C genome size (Mbp)	DNA index	Cycle value	Ploidy patterns (% distribution of nuclei)		
					2C	4C	8C
Donor plant							
Young leaves	2.76 ± 0.02	1349.64	0.29	0.26	74.56	17.84	7.60
Mature leaves	2.78 ± 0.02	1359.42	0.30	0.43	60.34	28.63	11.03
tTCL mediated organogenesis (TMO)-young leaves	2.75 ± 0.01	1344.75	0.29	0.29	78.80	16.0	5.20
Hardened plants (after 1 year)							
Young leaves	2.75 ± 0.02	1344.75	0.29	0.27	75.20	18.60	6.20
Mature leaves	2.77 ± 0.03	1354.53	0.30	0.37	62.14	29.0	8.86

* pg-Picogram; Mbp-Megabasepairs; 1 pg = 978 Mbp (Doležel et al. 2007); cell cycle value was calculated according to Barow and Meister (2003). Organs with cycle values above 0.1 are considered endopolyploid

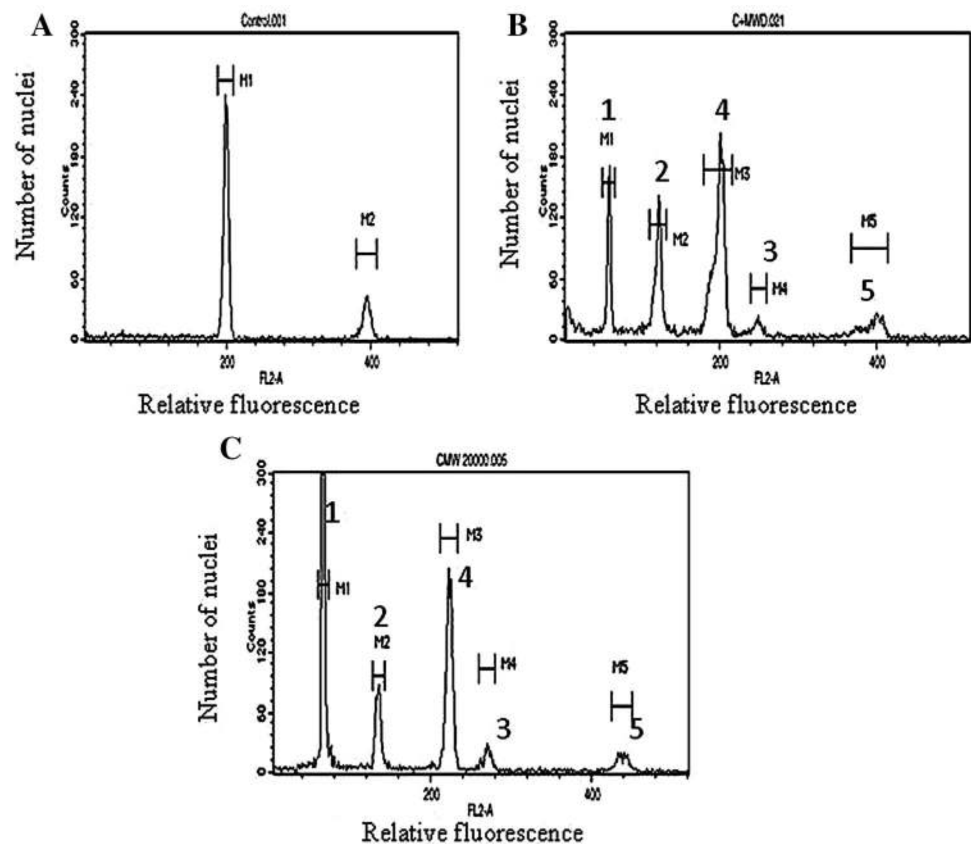

Fig. 3 Flow cytometric histogram of *M. wallichii*. **a** Reference standard (*P. sativum* Citrad), **b** Donor plant simultaneously chopped with internal standard **c** tTCL-derived plantlet with internal standard; 1: *M. wallichii* G0/G1 (2C) peak; 2: *M. wallichii* G2 (4C) peak; 3: *M. wallichii* 8C peak; 4: *P. sativum* G0/G1 peak and 5: *P. sativum* G2 peak

period indicates an up-regulation of plant defense mechanism against oxidative stress through the reduction of H_2O_2 into water (H_2O) and molecular oxygen (O_2) in peroxisomes (Mitrović et al. 2012). These results are in agreement with the findings of other workers (Faisal and Anis 2009; Jahan et al. 2014; Paul et al. 2014; Varshney and Anis 2012). A similar line of ROS detoxification has been observed in the activities of APX and GR where their levels have been increased during in vitro culture and maintained till second week of *ex*-vitro conditions (Fig. 4c, d). APX and GR are two important ROS scavengers of the ascorbate–glutathione cycle which scavenges H_2O_2 via NADPH-dependent reduction in chloroplasts, cytosol, mitochondria, apoplast and peroxisomes and thus

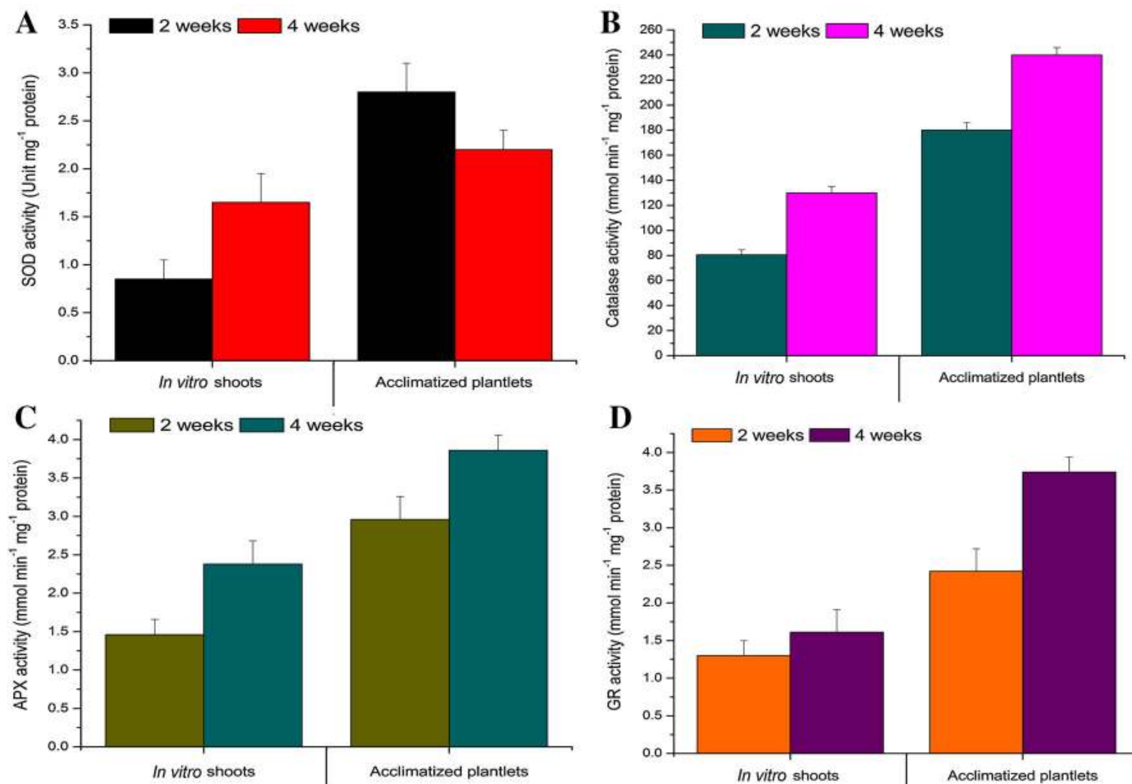

Fig. 4 Changes in the level of the antioxidant enzymes in in vitro and acclimatized plantlets of *M. wallichii*. **a** SOD, **b** CAT, **c** APX and **d** GR activity. Bars represent the mean ± SE

maintaining cellular redox under stress (Asada 1999; Mittler 2002). In the present findings, the intensity of the four antioxidative enzymes in the acclimatized plantlets was much higher than the in vitro plantlets which justify the ability of the plants with developed defensive machinery to cope up against oxidative stress during *ex-vitro* transfer. Present findings are in accordance with the studies reported on enhanced activities of antioxidant enzymes during in vitro culture and acclimatization in micropropagated plants of *Cymbidium giganteum* Wall., *Dendrobium fimbriatum* var. *oculatum, Rauvolfia tetraphylla* L., *Tylophora indica* (Burm. F.) Merr., *Cardiospermum halicacabum* L., *Vitex trifolia* L. and *Cassia alata* L. (Kumaria et al. 1990; Kumaria and Tandon 2000; Faisal and Anis 2009, 2010; Ahmed and Anis 2014a, b; Jahan et al. 2014).

Detection of H_2O_2 and lipid peroxidation during organogenesis and acclimatization

A continuous increase in endogenous H_2O_2 was observed during organogenesis and in the beginning of the acclimatization period but declined after 2 week clearly indicating stress tolerance in in vitro-regenerated plantlets of *M. wallichii* (Fig. 5a). The higher concentration of H_2O_2 might be due to induced oxidative stress generated during in vitro culture conditions. Moreover, recent research has found the active involvement of H_2O_2 as a secondary messenger molecule in regulating shoot primodium development and organogenesis (Gupta 2011; Guo et al. 2017). The change in the production of endogenous H_2O_2 was paralleled to that of the SOD activity which suggests proper functioning of the antioxidative enzyme machinery in the plant system. MDA, a lipoxidation end product of polyunsaturated fatty acids, is very often used as a suitable biomarker for lipid peroxidation to measure the level of oxidative stress (Bailly et al. 1996). A gradual decline in MDA content after 14 days of acclimatization period was observed in tTCL-derived plantlets indicating reduced oxidative stress (Fig. 5b). Similar results have been found in *Gerbera jamesonii* Bolus ex Hook. f. and *Abrus precatorius* L. (Chakrabarty and Datta 2008; Perveen et al. 2013).

Conclusion

An efficient, reproducible protocol has been developed for rapid and mass in vitro propagation via transverse thin cell layer (tTCL) culture from pseudobulbs of *M. wallichii*—a threatened terrestrial medicinal orchid of industrial

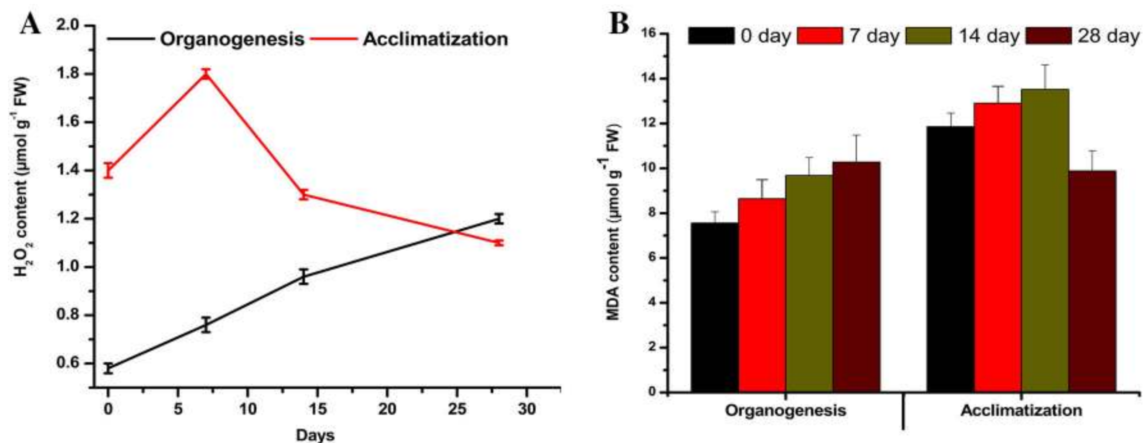

Fig. 5 Changes in H_2O_2 and MDA content during tTCL-organogenesis and acclimatization of *M. wallichii.* **a** H_2O_2 content, **b** MDA content. Bars represent the mean ± SE

importance. The frequency of adventitious shoot bud induction was greatly influenced by position and orientation of the tTCL explants and types and concentrations of PGRs applied. At same equimolar concentration, mT proved to be three times more superior to other conventional cytokinins in improving plant height and growth of this slow growing terrestrial orchid and positively influenced in vitro rooting and acclimatization. In vitro regenerated plants were analysed for their clonal fidelity by ISJ markers and FCM which revealed a stable genome size, ploidy and endoreduplication pattern as compared to donor plant. A significant up regulation in the activity of different antioxidant enzymes revealed the enhanced ability of in vitro-derived plantlets to cope up with the oxidative stress during in vitro and ex vitro conditions.

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Author contributions BB carried out the experiments, analyzed the data and drafted the manuscript. All the authors were involved in designing of the experiment. SK, HC and PT supervised the work. SK and HC edited the manuscript. All authors read and approved the final version of this manuscript.

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