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ORIGINAL PAPER

In vitro propagation of four saponin producing *Maesa* species

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Abstract A successful micropropagation system was developed for four different medicinal *Maesa* species. Multiple shoots were induced through both axillary bud formation and adventitious shoot regeneration from leaf explants. The explants were cultured on Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA), thidiazuron (TDZ) and/or α-naphthalene acetic acid (NAA). The success of regeneration varied for different species and depended on the type and concentration of plant growth regulators. Regenerated shoots spontaneously developed roots within 6 weeks on MS hormone-free medium. The rooted shoots were transferred to the greenhouse with a 100% success rate. Furthermore, flow cytometry analysis indicated that there were no changes in ploidy level of those regenerated shoots as compared with wild type adult plants. Thin layer chromatography (TLC) analysis revealed that common and distinguishing spot of saponins were similarly observed in regenerated shoots compared to the control plants. Therefore, the protocol also provides an effective means for the in vitro conservation of *Maesa* spp. that produce pharmaceutically interesting saponins.

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Introduction

Maesa argentea, M. balansae, M. lanceolata and *M. perlarius* (Myrsinaceae) are shrubs or small trees growing in the tropics of Africa and Asia. All four species play an important role in traditional medicine and ancient practices. For example, a paste of *M. perlarius* leaves is used in Chinese medicine to promote the healing of broken bones. In Cambodia, Laos and Vietnam, the roots of *M. perlarius* are used to stimulate digestion and urination and an extract of the leaves is used to cure measles (Wiart 2006). *M. balansae* is used for the treatment of allergies, sprains, anthelminthic infections, drunkenness and headache in the Northern part of Vietnam (Germonprez et al. 2004). The fruits of *M. argentea* are eaten and squeezed leaves are used as a fish poison in Nepal (Wiart 2006). *M. lanceolata* is well known in Rwandan traditional medicine, where the extracts of leaves and fruits are used against various diseases including hepatitis, dysentery, skin diseases and neuropathies (Sindambiwe et al. 1996).

Investigation of the methanol extract of dried leaves of different *Maesa* species resulted in the identification of triterpenoid saponin mixtures. In vitro bioactivity assays showed that *M. perlarius*, *M. balansae, M. lanceolata* and *M. argentea* have virucidal, molluscicidal, cytotoxic, haemolytic and anti-leishmanial actions (Sindambiwe et al. 1996; Foubert et al. 2008; Foubert et al. 2009). More extensive studies on *M. balansae* saponins have shown that these metabolites are effective in curing visceral leishmaniasis in mice and hamsters. The potency of the saponins is comparable to that of the commercial drug against

leishmaniasis, liposomal amphotericin B (Maes et al. 2004b; Maes et al. 2004a).

There are, however, difficulties accompanied with conventional propagation of *Maesa* species, e.g. the plants do not flower in greenhouse conditions. Therefore, in vitro tissue culture techniques have become a feasible alternative to improve the efficiency of propagation as well as to facilitate some in vitro experiments such as genetic transformation, protoplast fusion and investigation of the gene expression in saponin biosynthesis. In fact, in vitro culture has already been shown to be an efficient method for propagating medicinal plants (see reviews by (Debnath et al. 2006) and (Rout et al. 2000). Many plants are grown under in vitro conditions to conserve the germplasm, examples are micropropagation of *Cecropia* spp. (Nicasio-Torres et al. 2009), *Ceropegia intermedia* (Karuppusamy et al. 2009), *Bacopa monnieri* (Singh et al. 2009), *Asparagus racemosus* (Bopana and Saxena 2008) and *Searsia dentata* (Prakash and Van Staden 2008). In some cases, tissue and cell culture is also used for the production of interesting secondary metabolites. In vitro root cultures of *Catharanthus roseus* are used for the production of two anti-cancer alkaloids, vinblastine and vincristine (Ataei-Azimi et al. 2008). Cell cultures of *Taxus* spp. are used to produce large amounts of alkaloid taxol, which is a chemotherapeutic agent, approved in the treatment of a variety of cancers. Taxol is currently supplied through both a semi-synthetic process and plant cell culture (Vongpaseuth and Roberts 2007). In addition, cell cultures of *Panax ginseng* are used for the commercial production of triterpene saponins (Wu and Zhong 1999).

We are investigating the biosynthesis and production of the saponins in four different medicinal *Maesa* species. In this study, we present the optimization of an in vitro propagation method that greatly facilitates the production of genetically stable *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* plantlets that show vigorous growth in the greenhouse.

Materials and methods

Plant material

M. lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). *M. balansae* and *M. perlarius* seeds were collected in the Trang Dinh district, Vietnam by Nguyen Tap (National Institute of Medicinal Materials Lang Son Province). Finally, *M. argentea* seeds (no. 61-2068) were provided by the National Botanical garden (Meise, Belgium). The seeds were rinsed in 70% (v/v) ethanol for 30 s and subsequently surface sterilized with a 70% (v/v) solution of a commercial disinfection product (Haz-tabs; Guest Medical, Kent, UK). After three washes with distilled water, the seeds were placed on MS basal medium (Murashige and Skoog 1962) supplemented with 0.8% (w/v) agar (Lab M plant tissue culture agar MC29, Amersham) and 3% (w/v) sucrose (with pH 5.8). Seeds were germinated in a 16/8 h light/dark photoperiod at 26°C.

Axillary shoot formation

Three month old seedlings of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* were used as explant source for micropropagation through an axillary branching method. Shoots were excised, defoliated and implanted vertically into MS basal medium supplemented with 3% (w/v) sucrose and 0.15% (w/v) Gelrite (Marck & Co., Kelco Division, USA). For shoot multiplication, the basal medium was supplemented with 6-benzyladenine (BA) and 1-naphthaleneacetic acid (NAA) at different concentrations. The following concentrations were used: 4.4, 8.8, 13.2 and 22.2 μM **BA**, alone or in combination with 5, 10.7 and 13.5 μM **NAA** (Table 1). Shoots were incubated at 26°C in a 16/8 h light/dark period and axillary shoot formation was evaluated 8 weeks later.

Table 1 Influence of the plant growth regulators BA and NAA on axillary shoot multiplication in different *Maesa* species 8 weeks after incubation on multiplication medium

PGRs (μM)		*M. argentea*	*M. balansae*	*M. lanceolata*	*M. perlarius*
BA	NAA	# Shoots/explant (±SE)	# Shoots/explant (±SE)	# Shoots/explant (±SE)	# Shoots/explant (±SE)
–	–	1.0 ± 0.0^{d}	1.4 ± 0.3^{bc}	1.3 ± 0.2^{c}	2.2 ± 0.8^{abc}
4.4	–	3.0 ± 0.4^{bc}	2.2 ± 0.2^{ab}	1.7 ± 0.3^{c}	2.7 ± 0.5^{ab}
8.8	–	4.7 ± 0.3^{b}	2.7 ± 0.2^{a}	4.7 ± 0.7ab	2.8 ± 1.9^{bc}
13.2	–	6.7 ± 1.1^{a}	1.8 ± 0.5^{abc}	6.3 ± 1.2^{a}	3.2 ± 0.4^{a}
4.4	10.7	0.7 ± 0.2^{d}	1.3 ± 0.2^{bc}	0.8 ± 0.3^{c}	0.5 ± 0.2^{c}
13.2	13.5	0.0 ± 0.0^{d}	1.0 ± 0.0^{c}	1.2 ± 0.2^{c}	0.3 ± 0.2^{c}
22.2	5	1.5 ± 0.2^{cd}	2.0 ± 0.6^{abc}	2.8 ± 0.3^{bc}	3.8 ± 0.9^{a}

Different letters indicate significant differences ($P < 0.05$) within one species according to Duncan test

Adventitious shoot induction

Fully developed leaves from in vitro grown plants of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* were isolated and used as explants. Leaves (petiole was cut off from the leaf bases) were placed with the adaxial side in contact with MS basal medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. For shoot induction, auxin and cytokinins were added to the basal medium in different concentrations, either alone or in combinations. The following growth regulators were used in the given concentrations; 0.5, 1.35 and 2 μM **NAA**; 4.4, 13.3 and 22.2 μM **BA** and 4.5, 13.6 and 22.7 μM **thidiazuron** (TDZ) (Table 2). Leaves were incubated at 26°C with 16/8 h light/dark photoperiod. The average number of adventitious shoot induced per explant was recorded after 8 weeks of culture.

Rooting and acclimatization

Multiple shoots were formed through axillary branching and adventitious shoot induction. These shoots were isolated and transferred to basal MS medium lacking growth regulators and supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar, for elongation and rooting in one single step. Shoots were incubated in a 16/8 h light/dark photoperiod at 25°C.

Rooted plantlets were gently and thoroughly washed with water, to remove attached medium from the roots, and were transferred to 9×9 cm^2 small plastic pots containing a mixture of sand and peat soil (1:1). The plantlets were placed in a small greenhouse with a high humidity for 3 weeks to gradually acclimatize to greenhouse conditions.

Table 2 Effect of the plant growth regulators BA, TDZ and NAA on adventitious shoot regeneration from mature leaf explants of 4 *Maesa* spp. Shoot induction was evaluated and scored after 8 weeks; for each species, best result is indicated in bold

Plant growth regulators (μM)			*M. argentea*		*M. balansae*		*M. lanceolata*		*M. perlarius*	
BA	TDZ	NAA	# Shoots/explant (±SE)	Rooting	# Shoots/explant (±SE)	Rooting	# Shoots/explant (±SE)	Rooting	# Shoots/explant (±SE)	Rooting
–	–	–	0.0^{c}	No	0.0^{a}	No	0.0^{b}	No	0.0^{b}	No
4.4	–	–	0.0^{c}	No	0.0^{a}	No	0.0^{b}	No	0.0^{b}	No
13.3	–	–	0.0^{c}	No	0.0^{a}	No	0.0^{b}	No	0.0^{b}	No
22.2	–	–	0.0^{c}	No	0.0^{a}	No	0.0^{b}	No	0.0^{b}	No
4.4	–	0.5	0.0^{c}	No	0.0^{a}	No	0.0^{b}	No	0.0^{b}	Yes
4.4	–	1.35	0.0^{c}	No	0.0^{a}	Yes	0.0^{b}	Yes	0.0^{b}	Yes
4.4	–	2	0.0^{c}	No	0.0^{a}	Yes	0.0^{b}	Yes	0.0^{b}	Yes
13.3	–	0.5	0.0^{c}	No	0.0^{a}	Yes	0.0^{b}	No	0.0^{b}	Yes
13.3	–	1.35	0.0^{c}	Yes	0.0^{a}	Yes	0.0^{b}	Yes	2.3 ± 0.85^{a}	Yes
13.3	–	2	0.0^{c}	Yes	0.0^{a}	Yes	0.0^{b}	Yes	0.0^{b}	Yes
22.2	–	0.5	0.0^{c}	No	0.0^{a}	Yes	0.0^{b}	No	0.0^{b}	No
22.2	–	1.35	0.2 ± 0.22^{c}	Yes	0.0^{a}	Yes	0.0^{b}	No	0.0^{b}	No
22.2	–	2	0.0^{c}	Yes	0.0^{a}	Yes	0.0^{b}	Yes	0.3 ± 0.33^{b}	No
–	4.5	–	0.0^{c}	No	0.0^{a}	No	0.0^{b}	No	0.0^{b}	No
–	13.6	–	0.0^{c}	No	0.0^{a}	No	0.0^{b}	No	0.0^{b}	No
–	22.7	–	1.7 ± 0.85^{bc}	No	0.0^{a}	No	0.0^{b}	No	0.0^{b}	No
–	4.5	0.5	0.4 ± 0.34^{c}	No	0.6 ± 0.34^{a}	Yes	0.0^{b}	No	**3.6 ± 0.62**^{a}	No
–	4.5	1.35	4.9 ± 2.98^{b}	No	0.0^{a}	Yes	0.0^{b}	No	0.0^{b}	No
–	4.5	2	0.4 ± 0.44^{c}	No	0.0^{a}	Yes	0.0^{b}	Yes	0.0^{b}	Yes
–	13.6	0.5	0.7 ± 0.37^{c}	No	0.0^{a}	Yes	0.0^{b}	No	0.0^{b}	No
–	13.6	1.35	5.0 ± 1.24^{b}	No	0.0^{a}	Yes	1.3 ± 0.57^{ab}	No	0.0^{b}	No
–	13.6	2	**9.7 ± 2.06**^{a}	No	0.0^{a}	Yes	0.0^{b}	Yes	0.0^{b}	Yes
–	22.7	0.5	5.4 ± 0.93^{b}	No	0.0^{a}	Yes	0.0^{b}	No	0.0^{b}	No
–	22.7	1.35	2.6 ± 0.94^{bc}	No	0.0^{a}	Yes	**4.1 ± 1.58**^{a}	No	0.0^{b}	No
–	22.7	2	1.6 ± 0.84^{bc}	No	**1.2 ± 1.2**^{a}	Yes	1.9 ± 1.2^{ab}	Yes	0.0^{b}	Yes

Different letters indicate significant differences ($P < 0.05$) within one species according to Duncan test

Flow cytometry

Flow cytometry was performed to check ploidy of the regenerated shoots. Leaf samples derived from both micropropagation through axillary branching and adventitious shoot from leaf explants were compared with wild adult plants. Approximately 50–100 mg of both young in vitro and wild adult plant leaf material was chopped with a razor blade in 2 ml Galbraith buffer (45 mM $MgCl_2$, 20 mM 3-(N-morpholino)propanesulfonic acid (MOPS), 30 mM sodium citrate and 0.1% (v/v) Triton X-100) (Galbraith et al. 1983; Loureiro et al. 2006) to isolate nuclei. 5% (w/v) of Polyvinylpyrrolidone 1000 (PVP-10) was also added to the extraction buffer to neutralize interference of cell metabolites in the measurements. The nuclei suspension was filtered through a 50 μm strainer to remove debris. Nuclei were stained by mixing 400 μl of nuclei suspension with 50 μl propidium iodide (0.5 mg/ml). 50 μg/ml RNAse was added to prevent staining of double-stranded RNA. The DNA content of the isolated samples was measured by using a Beckman Coulter EPICS® Altra™ Flow Cytometer. The instrument was equipped with a 15 mW 488 nm air-cooled argon-ion laser. Fluorescence was detected through a 575 nm band-pass filter. Disintegrated nuclei and other cell debris signals were eliminated from analysis by two gating systems; forward scatter based on nuclei proportional and PMT3 based on propidium iodide fluorescence. The gates were consistently maintained for all samples in each run and the resulting PMT4 histograms were analyzed using EXPO™32 MultiCOMP software (Beckmann Coulter). Together with each leaf sample, leaf tissue from diploid (2n) *Arabidopsis thaliana* was included as an internal reference standard.

Saponin extraction and analysis

For saponin extraction, 50 mg of plant material was grinded with liquid nitrogen. 250 μl of 50% (v/v) methanol was added and samples were sonicated for 1 h. Subsequently, extracts were centrifuged at 2,000 rpm for 10 min and the supernatant was transferred to a fresh eppendorf tube. The pellet was resuspended with 250 μl 50% (v/v) methanol and sonicated for another hour. Samples were centrifuged at 2,000 rpm for 10 min and the supernatant was combined with the supernatant from the first step. Samples were dried for 3–4 h using a vacuum concentrator (Heto VR-I, High Technology of Scandinavia) attached to a Savant RT4104 refrigerated condensation trap. Afterwards, the pellet was resuspended in 50 μl 80% (v/v) methanol. This extract was further used for TLC analysis.

TLC analysis was performed with normal phase silica gel 60 plates with fluorescence indicator (F_{254}) (Merck KGaA, Germany). For the mobile phase, the upper layer of a n-butanol/acetic acid/H_2O (40/10/50) was used. One hour before starting the TLC run, the mobile phase was brought into the TLC tank and a filter paper was placed at the back of the tank. 10 μl of the samples was spotted at 2 cm from the sides and bottom of the plate. Afterwards, the plate was placed in the tank for 5 h. The saponins were stained using an anisaldehyde reagent (5 ml/l p-anisaldehyde, 100 ml/l acetic acid, 850 ml/l methanol and 50 ml/l sulphuric acid). The reagent was sprayed onto the TLC plate using an EcoSpray (Carl Roth GmbH). Saponin spots were visible 10 min after heating the plate to 100°C on a hot plate.

Statistical analysis

Each petri dish and bottle with four or five explants was considered as one replicate. The presented experiments are comprised of at least three replicates. Number of shoots was recorded 8 weeks after induction both for axillary shoot formation and adventitious shoot from leaf explants. The data were analyzed by one-way ANOVA followed by Duncan test ($P < 0.05$).

Results and discussion

Micropropagation through axillary shoot formation

The experiments conducted to optimize the medium for multiplication of *M. argentea, M. balansae, M. lanceolata* and *M. perlarius* are summarized in Table 1. The effect of different hormones on the induction of shoots was clearly dependent on the species used. For *M. argentea* and *M. lanceolata* the highest number of shoots, 6.7 and 6.3 respectively, was achieved when using BA alone at a concentration of 13.2 μM. *M. balansae* responded best to 8.8 μM BA with 2.7 shoots per explant. Other hormone treatments did not show a significant difference with the control. For *M. perlarius* none of the hormone treatments stimulated shoot induction and no difference with the hormone free control medium was observed. Noticeably, the multiplication rate of the control plants of *M. perlarius* was higher than that of the controls of the other species.

For *M. argentea, M. balansae* and *M. lanceolata* the number of shoots per explant was always highest when treated with BA alone. Addition of NAA had a negative effect on the number of shoots formed. This is in contrast with the results published for *Maesa ramentacea*, where a synergistic action between BA and NAA was observed (Kanchanapoom and Boonvanno 2000). It was also noted that for *M. argentea, M. lanceolata* and *M. perlarius* the highest concentration of BA induced highest number of shoots; therefore it is possible that concentrations higher than 13.2 μM of BA could be even more effective. Though,

it should be considered that high concentrations of BA can be toxic to the plants through induction of programmed cell death (Carimi et al. 2004).

Adventitious shoot induction

To obtain adventitious shoot induction, leaf explants were incubated on MS medium supplemented with various concentrations of either BA or TDZ, individually or in combination with NAA. For this experiment, two different types of cytokinins were chosen; BA is a purine-type cytokinin, while TDZ is a phenylurea with cytokinin like actions. Adventitious shoots were observed for all four *Maesa* species; however, optimal shoot induction conditions differed for the analyzed species (Table 2). Generally, small outgrowths were induced at the surface of leaf explants after 4–5 weeks of culture. No shoots were induced on hormone-free medium or when cytokinins alone were added to the culture medium, implying that a combination of cytokinin and auxin was necessary for adventitious shoot formation in *Maesa* leaf explants. The only exception is shoot induction on *M. argentea* leaves with the highest concentration of TDZ.

Application of BA in combination with NAA resulted in very low frequency shoot formation for two out of the four *Maesa* species. For *M. argentea*, shoot induction was only achieved with 22.2 μM BA and 1.35 μM NAA (0.2 shoots per explant). For *M. perlarius* two combinations of BA and NAA, namely 13.3 μM BA with 1.35 μM NAA and 22.2 μM BA with 2 μM NAA induced adventitious shoots, with a mean of 2.3 and 0.3 shoots per explant, respectively. For all four *Maesa* species, treatment of leaves with BA in combination with NAA often lead to root induction, which was not observed when BA alone was used.

In contrast to the results of shoot induction using BA in combination with NAA, the combination of TDZ and NAA induced multiple adventitious shoots on all four *Maesa* species. This observation is suggestive for a synergetic effect of TDZ and NAA. For *M. argentea*, the highest average number of shoots (9.7 shoots per explant) was obtained when leaf explants were incubated on MS medium supplemented by 13.6 μM TDZ and 2 μM NAA (Fig. 1a). Except for the two lower concentrations of TDZ alone (4.5 and 13.6 μM), all the combinations and concentrations of TDZ and NAA resulted in shoot induction for *M. argentea*. For the other species shoots were also observed, however, not so frequently as for *M. argentea*. *M. lanceolata* leaves showed shoot induction with three combinations of TDZ and NAA, with a maximum of 4.1 shoots per explant when 22.7 μM TDZ was combined with 1.35 μM NAA (Fig. 1c). *M. perlarius* leaf explants developed adventitious shoots with only one combination, 4.5 μM TDZ and 0.5 μM NAA, with a mean of 3.6 adventitious shoots per explant (Fig. 1d). *M. balansae* showed a lower frequency of shoots regeneration with at maximum 1.2 shoots per explant when the highest concentrations of TDZ and NAA were combined (22.7 and 2 μM, respectively) (Fig. 1b). A combination of 4.5 μM TDZ and 0.5 μM NAA also induced shoots, however, at a very low number (0.6 shoots per explant). Except for *M. argentea*, all species showed root formation in addition to shoot formation.

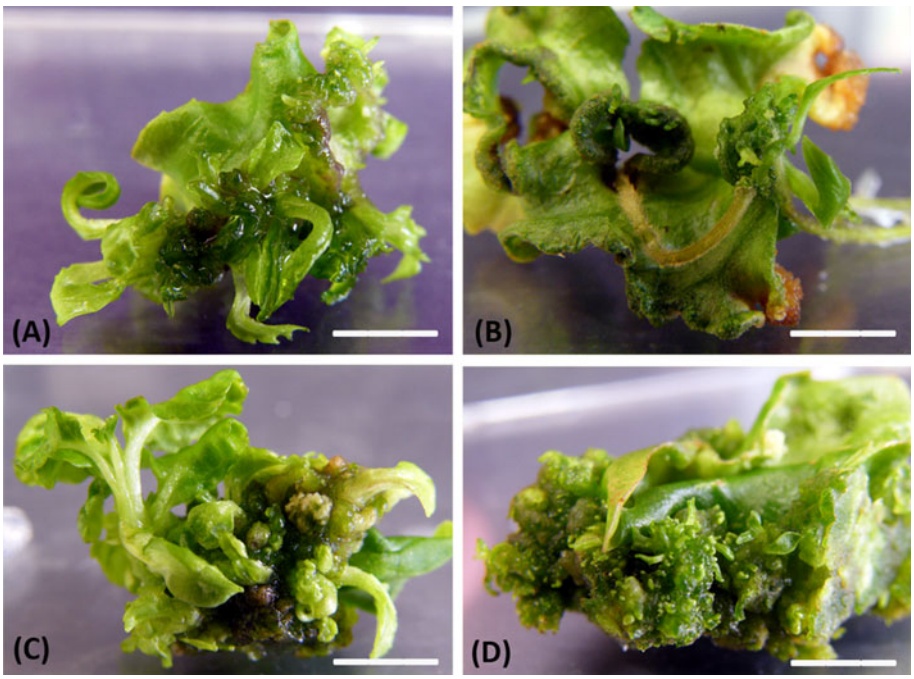

Fig. 1 Induction of adventitious shoots on leaf explants of **a** *M. argentea*, **b** *M. balansae*, **c** *M. lanceolata* and **d** *M. perlarius*. Pictures were taken 8 weeks after culture on MS medium supplemented with TDZ and NAA. *Bar* = 0.5 cm

Taken together, these results suggest that TDZ acts synergistically with NAA to promote shoot induction. In contrast to addition of BA and NAA, TDZ played an essential role in inducing adventitious shoot induction on leaf explants from *Maesa* in vitro plants and proved to be the more effective cytokinin in our study. The higher effectiveness of TDZ as compared to BA, for induction of adventitious shoots from leaf explants, was also reported for other plant species such as *Mimulus aurantiacus* (Murovec et al. 2010), *Lysimachia* spp. (Zheng et al. 2009), *Paulownia tomentosa* (Corredoira et al. 2008) and *Echinacea* purpurea (Jones et al. 2007). Thidiazuron, a synthetic phenylurea derivative, is one of the most active cytokinin-like compounds for woody plant tissue culture (Huetteman and Preece 1993; Lu 1993). Unlike classic cytokinins, TDZ is competent of fulfilling both the cytokinin and auxin requirement of various regenerative responses of many different plant species (Jones et al. 2007). A low concentration of NAA was necessary to induce direct shoot regeneration from leaf explants. This means that NAA can be considered as a critical growth regulator for shoot regeneration of *Maesa* spp. De Gyves and coworkers hypothesized that there is a synergism existing between TDZ and both endogenous and exogenous auxin (De Gyves et al. 2001). This finding corresponds with our results that in general the combination of TDZ and NAA promoted more shoots compare to application of TDZ alone. The combination of TDZ and NAA has also been reported to induce shoot regeneration from leaf explants of several plant species (Zhou et al. 2010; Espinosa et al. 2006; Feng et al. 2010).

Rooting and acclimatization

Regenerated shoots elongated and developed roots in basal MS media without phytohormones with an efficiency of 100%. Generally, *Maesa* roots started to emerge within 2 weeks. Plantlets spontaneously produced well developed root system within 6 weeks on hormone-free medium (Fig. 2a–d). The very efficient rooting mechanism of these plants provides an additional advantage for the rapid clonal propagation without any growth regulators needed. This spontaneous root formation is effective during the establishment of the plantlets in soil as well. Rooting of shoots without any addition of hormones is also reported for *Vitis champinii* (Mukherjee et al. 2010), *Tuberaria major* (Gonçalves et al. 2010), *Drymaria cordata* (Ghimire et al. 2010) and *Aloe polyphylla* (Bairu et al. 2007). However, most species require auxin treatment for root induction prior to acclimatization.

For acclimatization, regenerated and rooted shoots from all explants of *Maesa* spp. could be hardened with a 100% efficiency rate. After transfer to greenhouse condition, the plantlets continued to grow and developed into normal and vigorous plants (Fig. 2e–h).

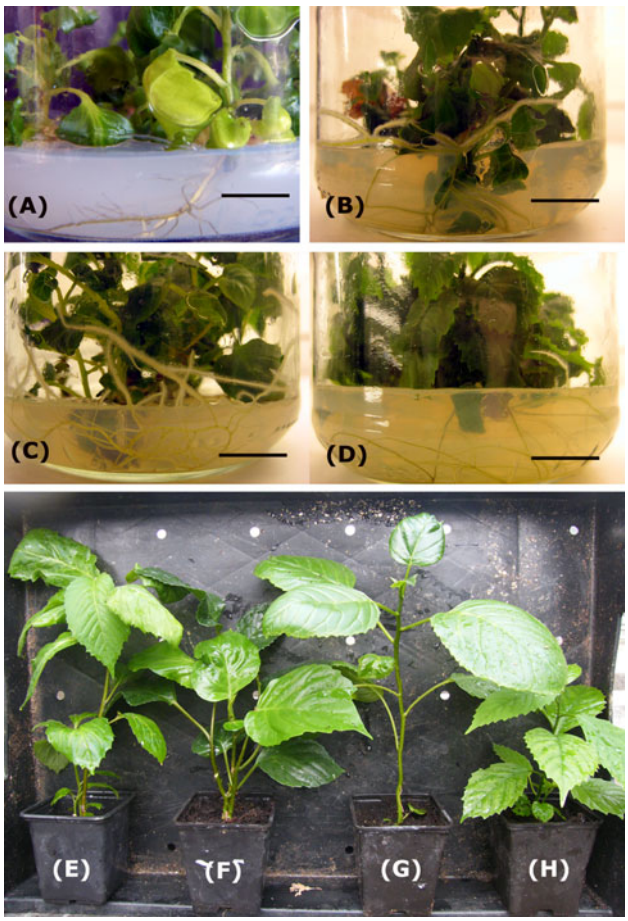

Fig. 2 Rooting of in vitro regenerated shoots of **a** *M. argentea*, **b** *M. balansae*, **c** *M. lanceolata* and **d** *M. perlarius*. Root pictures were taken 8 weeks after culture on MS basal medium. Regenerated shoot acclimatized for 4 months in greenhouse conditions of **e** *M. argentea*, **f** *M. balansae*, **g** *M. lanceolata* and **h** *M. perlarius*. *Bar* = 1 cm

Stability of regenerated shoots at ploidy level

Inducing adventitious shoots through in vitro regeneration is sometimes accompanied by genetic instability through a process known as somaclonal variation (Larkin and Scowcroft 1981). To further investigate the impact of tissue culturing on the plant genetic stability, shoots regenerated through the application of different types and concentrations of plant growth regulators and from different types of explants were analyzed. Since this regeneration process escapes the normal plant fertilization and development, it is also possible that the variation occurs by epigenetic factors (Kaeppler et al. 2000). The variability that is commonly noticed are the ploidy level, chromosome structure, mitotic abnormalities and other cytological disorders (Radić et al. 2005). Flow cytometry is considered as a powerful tool for

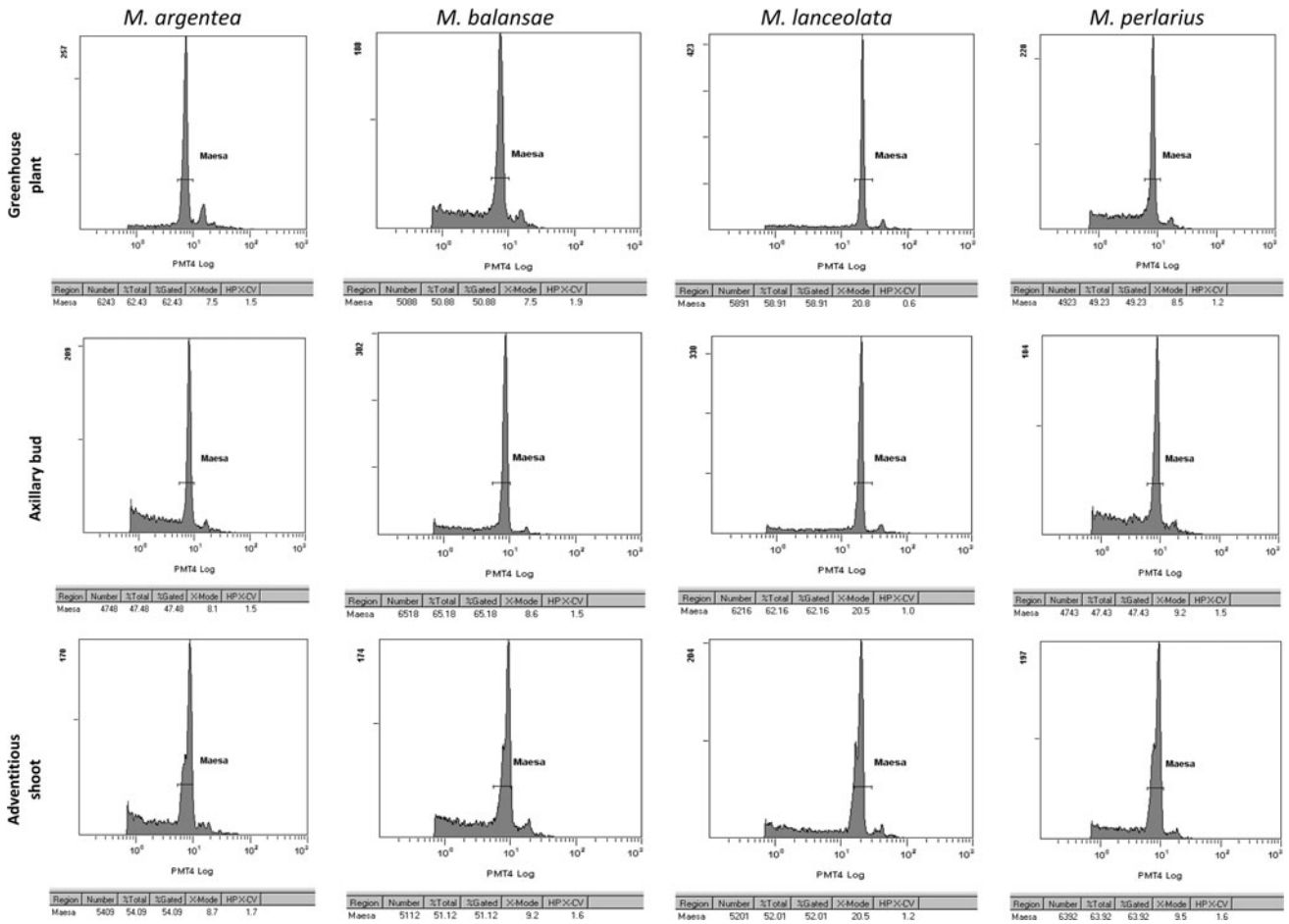

Fig. 3 *Histogram* of relative fluorescence intensity (log-transformed, PMT4 log) of isolated nuclei from 4 *Maesa* species

estimating DNA ploidy level in plant in vitro (Cousin et al. 2009) and has already been used to investigate the genetic variability of in vitro regenerated shoots (Vujovic et al. 2010; Mallón et al. 2010; Obae and West 2010).

The ploidy level of *Maesa* regenerated shoots was determined through analyzing small pieces of leaves. The relative nuclear DNA content of *Maesa* spp. was determined for different plant categories: greenhouse plants grown from seedling, plantlets grown from axillary buds and adventitious shoots from leaf explants. The flow cytometry analysis showed the similar peaks in all samples within every *Maesa* species (Fig. 3). No significant differences in DNA content were observed for each of the in vitro cultivated *Maesa* plant material indicating that no changes in ploidy level occurred during the regeneration process. In addition, early stage of regeneration process from leaf explants stimulated by hormones was also analyzed to check for ploidy level changes. Also in this plant material we did not observe deviations in DNA content, indicating that the hormone treatment did not alter the ploidy level of the plants.

Saponin production in regenerated shoots

Because we are interested in the saponins produced by *Maesa* species, it is important to assess the capacity to produce saponin in in vitro cultivated plants. To investigate the saponin content, extracts from leaves of regenerated plantlets by axillary shoot formation and adventitious shooting of all four *Maesa* species and of the corresponding in vitro control plants were compared using thin layer chromatography (TLC) (Fig. 4). Using purified *M. lanceolata* saponins (sample MC3B1) as a reference standard, qualitative TLC analysis showed that regenerated shoots produced a phytochemical profile similar to shoots of greenhouse plants. Saponin from *M. lanceolata* was characterized by the appearance of single spot at $R_f = 0.17$. *M. balansae* and *M. perlarius* produced a similar profile consisting of a major spot with $R_f = 0.16$ and a serious of minor spots with lower R_f values. These minor compounds were not observed in the *M. lanceolata* extracts. In the previous study, liquid chromatography-mass spectrometry (LC–MS) analysis also showed that *M. perlarius* produced

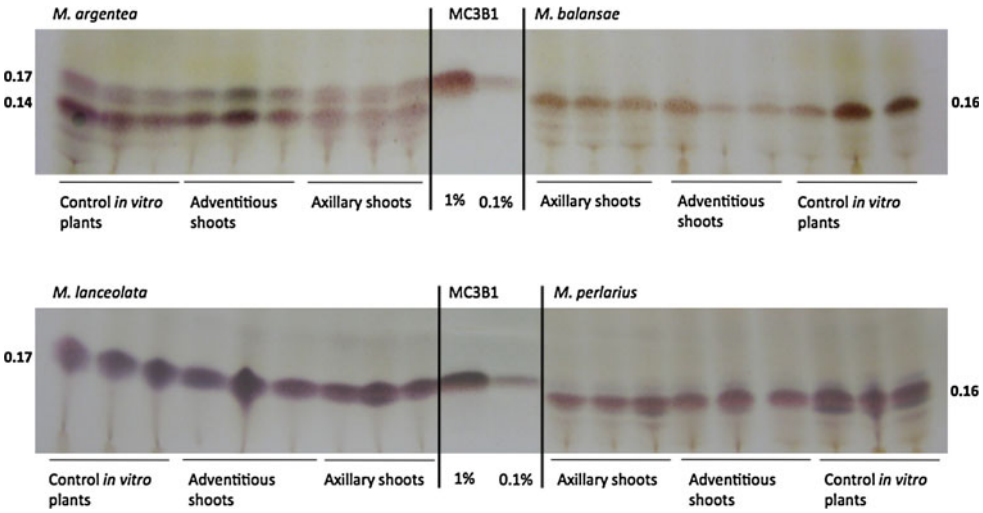

Fig. 4 TLC analysis of saponin production in control in vitro plants and plantlets induced through axillary shoots and adventitious shoots regeneration for **a** *M. argentea*, **b** *M. balansae*, **c** *M. lanceolata* and **d** *M. perlarius* with their *Rf* values respectively. MC3B1 is an HPLC purified saponin mixture of *M. lanceolata* saponins and is used here as a reference sample 0.1 and 1% (w/v)

saponins with the same retention time and molecular weight as the maesabalides, saponin from *M. balansae* (Foubert et al. 2009). *M. argentea* showed a different profile with two major compounds with $R_f = 0.14$ and 0.17. The presence of a spot at $R_f = 0.17$ is in agreement with the LC–MS results showing that *M. argentea* leaves contained maesasaponin I, III.2, IV.3, V.3 and VI.2. (Foubert et al. 2009). On the other hand, a spot at $R_F = 0.14$ suggests that *M. argentea* produces saponins quite distinct from the maesasaponins described for *M. lanceolata.*

We also analyzed the saponin content in hairy roots and undifferentiated callus (Faizal and Geelen, unpublished results). In this in vitro material, the saponin content was about 10 fold lower than what is found in greenhouse shoots and roots. Although the saponin detection method applied here does not allow a solid assessment of the amount of saponin produced, we are confident that, compared to hairy roots and callus, the in vitro regenerated shoots had maintained a significant capacity to produce and accumulate saponins. Regenerated shoots that were acclimatized and grown in the greenhouse for several weeks accumulated saponins at a concentration similar to what was detected in plants that were cultivated over long periods (data not shown). Together, the results show that in vitro grown shoots are similar to ex vitro plants with regard to morphological characteristics and saponin biosynthesis.

Conclusion

This report provides an efficient and reproducible protocol for adventitious and axillary shoot regeneration of the species *M. argentea, M. balansae, M. lanceolata* and *M. perlarius*. The regenerated plants are of the same ploidy as the parental material and show a normal level of saponin production. The established protocol will be useful not only for the micropropagation and conservation of *Maesa* spp., but also as a potential system for genetic modification of these medicinal plants.

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