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Full Length Research Paper

High efficiency protoplast isolation from *in vitro* cultures and hairy roots of *Maesa lanceolata*

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***In vitro* cultures of the medicinal plant *Maesa lanceolata* were established to enable the cultivation of plant material for the production of protoplasts. Callus cultures were initiated using leaves collected from shoot cultures and the root tips from hairy root cultures obtained upon *Agrobacterium rhizogenes* transformation. For the isolation of protoplasts, the different explant material of *M. lanceolata* was exposed to an enzyme mixture consisting of 1.5% cellulase, 0.5% macerozyme R-10 and 0.5 M mannitol. About 6 x 10^6 protoplasts g^{-1} fresh weight were obtained from leaf material and 5 x 10^5 protoplasts g^{-1} fresh weight from callus. To obtain high amounts of hairy root protoplasts, the cultures were pre-treated with the auxin indole-3-butyric acid (IBA) that stimulated the formation of novel root tips. Using the dissected root tips as starting material, 8 x 10^5 protoplasts g^{-1} fresh weight were obtained per preparation. The protoplast isolation method will enable further studies on the transformation and fusion of protoplasts from *M. lanceolata*.**

Key words: *Maesa lanceolata*, *in vitro* conservation, tissue culture, protoplast isolation.

INTRODUCTION

Saponins are glycosides of steroidal or triterpenoid polycyclic structures with distinctive foaming characteristic. They are produced by a wide variety of plant species as a defence mechanism against fungal, insect or herbivore attack (Francis et al., 2002; Papadopoulou et al., 1999). Although in general saponins have strong haemolytic activity, several types have been shown to possess medicinal properties, e.g. the ginseng saponins from *Panax ginseng* (Sparg et al., 2004).

Species of the genus *Maesa (Maesaceae)* produce saponins that are potentially interesting for the pharmaceutical industry. *M. lanceolata* is used in traditional medicine as a cure for Leishmania, e.g. in Rwanda, but it is also used for fishing by several tribes in Congo (Bagalwa and Chifundera, 2007; Maes et al., 2004b; Sindambiwe et al., 1996). Bioassay guided fractionation of the methanol extract of dried leaves of *M. lanceolata* resulted in the isolation of a triterpenoid saponin mixture (Sindambiwe et al., 1996). This mixture had moderate virucidal and high haemolytic activity and showed a severe toxic effect on snails (Sindambiwe et al., 1998). Moreover, maesa saponins showed anti-angiogenic activity, which could be useful in the treatment of certain types of tumours (Apers et al., 2002). Currently, *M. lanceolata* is not cultivated and its medicinal uses are based on wild collecting. In the absence of conventional breeding facilities, methods based on *in vitro* tissue culture offer a valuable alternative to improve and manipulate the production of saponins. Here, we describe a highly efficient protoplast formation method for *M. lanceolata*, with the aim to generate a vehicle for transformation and somatic hybridisation.

During the last decades, the isolation, fusion and culture of protoplasts has been described for a diverse range of plant species (Davey et al., 2005), including the saponin producing alfalfa (*Medicago sativa*) (Jin et al., 2003), common bulb onion (*Alium cepa*) (Karim and Adachi, 1997), camphor tree (*Cinnamomum camphora*) (Du and Bao, 2005), date palm (*Phoenix dactylifera*)

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Abbreviations: 2,4-D, 2,4-Dichlorophenoxyacetic acid; **BA,** N_6-benzylaminopurine; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **NAA,** α-naphthalene acetic acid; **MS,** Murashige and Skoog.

Table 1. The effect of different hormone combinations on callus induction on leaf and hairy root material of *M. lanceolata*, after 6 weeks of culture.

Growth regulators (µM)				Callusing on leaves	Morphology of leaf callus	Callusing on hairy roots	Morphology of hairy root callus
NAA	BA	2,4-D	Kinetin				
-	-	-	-	no		no	
2.7	-	-	-	no		no	
-	4.4	-	-	no		no	
-	-	5	-	yes	SY	yes	SY
-	-	-	0.46	no		no	
2.7	4.4	-	-	no		no	
2.7	-	5	-	yes	SY	no	
2.7	-	-	0.46	no		no	
-	4.4	5	-	yes	HB	no	
-	4.4	-	0.46	no		no	
-	-	5	0.46	yes	HB	no	

Experiments were performed in triplicate and callus induction was always observed in each replicate. **SY,** Smooth, yellow callus; **HB,** hard brownish callus.

(Chabane et al., 2007) and red cabbage (*Brassica oleracea*) (Chen et al., 2004). A few studies report a change in secondary metabolism after somatic hybridisation. One example is the formation of novel glycoalkaloids in somatic hybrids between cultivated and wild potato species. Fusion of protoplasts of *Solanum brevidens* and *Solanum tuberosum* led to production of secondary metabolites that were not found in both parents (Laurila et al., 1996). The same phenomenon was observed by Saverese and co-workers when analysing the glycoalkaloid content of somatic hybrids of *Solanum tuberosum* and *S. bulbocastanum* (Savarese et al., 2009). Previously, we developed method for the gene-ration and conservation of hairy roots (Lambert et al., 2009). The objectives of this study were to establish *M. lanceolata* callus and shoot cultures, which can be used for *in vitro* propagation of this promising medicinal plant, to analyze protoplast yield starting from different types of *in vitro* cultures and to obtain fluorescent protoplasts that will facilitate the process of protoplast fusion and selec-tion of fusion products.

MATERIALS AND METHODS

Plant material

M. lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). 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 Murashige-Skoog (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.

Hairy root induction

M. lanceolata hairy roots were induced using *Agrobacterium rhizogenes* (strain LBA 9402/12) transformation on leaf discs. For the selection of the transgenic material, GFP was used as a visible marker. The hairy roots were induced by wounding the leaf material and 3 days of co-cultivation with the *Agrobacteria*. After co-cultivation the leaves were placed on solid Murashige-Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 0.8% (w/v) agar, 2% sucrose and 500 mg/L cefotaxime (Duchefa, The Netherlands) to arrest *Agrobacteria* growth. Hairy roots were isolated from the leaf material after 15 - 30 days and were placed on solid Schenk and Hildebrandt (SH) basal medium (Schenk and Hildebra 1972) supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose. Hairy root cultures were cultured in the dark at 25°C and were subcultured every month.

Callus induction

Leaves of *in vitro* grown *M. lanceolata* were used as explants for callus induction. The leaves were isolated and cut into slices of approximately 0.5 cm and were then put on MS basal medium supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose (with pH 5.8). For callus induction, auxins and cytokinins were added to the basal medium in different combinations and the effect of different hormones and combinations of hormones on callus induction was studied. The following growth regulators were used in the given concentration; 2.7 µM 1-naphthalene acetic acid (NAA), 4.4 µM 6-benzylaminopurine (BA), 5 µM 2,4-dichlorophenoxy acetic acid (2,4-D) and 0.46 µM kinetin (Table 1). Leaf material was incubated at 25°C in the dark and callus formation was scored 6 weeks later. After evaluation, callus was put on fresh medium with the same composition as for the callus induction.

Alternatively, GFP positive *M. lanceolata* hairy roots were used as explants to obtain GFP fluorescent callus. Root tips of approximately 1 cm were isolated and put on SH basal medium supplemented with 0.8% (w/v) agar and 3% (w/v) sucrose (pH 5.8). For induction of callus the same combinations and concentrations of hormones were used as for induction of callus on leaf material (Table 1).

Protoplast isolation

In order to find the best conditions for protoplast isolation, different enzyme mixtures were tested. The mixture always consisted of enzymes, a sugar alcohol (mannitol or sorbitol) and 5 ml of distilled water. Four different enzymes were used in the given concentrations; 1, 1.5 or 2% (w/v) cellulase onnozuka RS (Duchefa Biochemie BV, The Netherlands), 1.5% cellulase Onnozuka R-10 (Duchefa Biochemie BV, The Netherlands), 0.5, 1 and 1.5% (w/v) macerozyme R-10 (Duchefa Biochemie BV, The Netherlands) and 0.05 or 0% (w/v) pectolyase Y-23 (Duchefa Biochemie BV, The Netherlands). Mannitol was used in a concentration of 0.5, 0.6, 0.7 or 0.8 M, sorbitol was used in a concentration of 0.5, 0.6 or 0.7 M.

The protocol for protoplast isolation from callus induced on leaves and callus induced on hairy roots was exactly the same. 500 mg of 1 week old callus was transferred to 5 ml of filter-sterilized enzymatic mixture. For the isolation of protoplasts from *M. lanceolata* leaves, *in vitro* plants of 6 - 12 months old were used. 100 mg of leaf material was transferred to 5 ml of enzymatic solution.

To enhance the yield of protoplasts when using *M. lanceolata* hairy roots as starting material, the roots were treated with 5 µM indole-3-butyric acid (IBA) one week before protoplast isolation. The root tips of the hairy roots were removed and placed on medium containing IBA or on medium without growth regulator. After 24 h the hairy roots were transferred to basal SH medium without any growth regulators. One week later, 100 mg of the hairy roots was transferred to 5 ml of enzymatic solution.

Incubation in the enzyme mixtures with cellulase R-10 was performed overnight (~ 15 h), in the dark at 25°C and on a rotary shaker at 50 rpm. Incubation in the enzyme mixtures with cellulase RS was performed during 5 h, in the dark at 25°C and on a rotary shaker set at 50 rpm. After incubation, the protoplast - enzyme mixture was filtered through a 40 µM nylon filter (BD Biosciences Europe) and centrifuged for 10 min at 500 rpm. The supernatant was discarded and the pellet was resuspended in a washing solution (1 x MS salts and 0.5 M mannitol) and centrifuged for 5 min. After another wash following the above procedure, the centrifugation was repeated. Finally the protoplasts were resuspended in 1 ml of washing solution.

A Fuchs-Rosenthal haemocytometer was used to count protoplasts and an inverted Olympus (IX-81) microscope was used to confirm fluorescence of transgenic tissue and to measure the diameter of the protoplasts (using Cell^{M} software, Olympus).

RESULTS

Hairy root induction

M. lanceolata hairy roots appeared from wounded sites in leaf discs, 15-30 days after inoculation with *A. rhizogenes* strain LBA 9402/12. Uninfected control explants did not form adventitious roots. GFP was used as a visible marker to select transformed hairy roots. Isolated hairy roots, in contrast to untransformed roots, grew autonomously in hormone free medium. Hairy root cultures were grown in the dark at 25°C on solid medium and subcultured every month.

Callus induction

Tested hormone combinations for callus induction on leaf and hairy root material are represented in Table 1. Six weeks after induction, callus was observed as well on leaves as on hairy roots (Table 1). Results indicate that the auxin 2,4-D is essential for callus induction for both types of explants. On leaf material, callus was observed when 2,4-D was applied alone or in combination with NAA, BA or kinetin. For callus induction on hairy roots 2,4-D alone was sufficient to induce 100% callusing.

The induced calli showed morphological variation and two types of calli were observed: smooth, yellow callus and harder, brownish callus. Remarkable was that the morphology of the callus was dependent on the type of growth regulators that were used. Calli induced by auxins (2,4-D alone or in combination with NAA) showed the smooth, yellow morphology. On the contrary, calli induced by auxin in combination with cytokinin (2,4-D in combination with BA or kinetin) displayed the hard, brownish phenotype. Callus induced on hairy roots also showed strong GFP fluorescence.

Callus was subcultured every month and put on fresh medium with the same composition. For protoplast isolation, callus of 1 week post subculturing was used.

Protoplast isolation

Protoplast isolation from callus and leaf material

The first material tested for protoplast isolation was *M. lanceolata* callus induced on leaves. Small scale preliminary tests were performed to find conditions required for efficient protoplast isolation. The basic enzyme mixture used consisted of cellulase RS, macerozyme R-10, pectolyase Y-23 and mannitol. We varied cellulase, macerozyme, pectolyase and mannitol conditions. In addition, we tested sorbitol as osmoprotectant and we tested if shaking during protoplast isolation was beneficial. Callus was incubated for 5 h in the enzymatic solution to allow subsequent manipulation of the protoplasts within a single day. The results are shown in Figure 1. Based on the preliminary tests, the following enzyme mixture, comprising 1.5% cellulase RS, 0.5% macerozyme R-10 and 0.5 M mannitol was used for further experiments. Samples were put on a rotary shaker at 50 rpm during isolation. Using this protocol an average of 2.7 x 10^5 protoplasts g^{-1} fresh weight was achieved (Table 2).

Because of practical reasons we also tried to adjust the protocol in such a way that we could do the protoplast isolation overnight instead of 5 h. 1.5% cellulase R-10 was used instead of 1.5% cellulase RS and isolation took place during the night for 15 h. The incubation conditions were not changed. This isolation protocol yielded two times more protoplasts, with a mean of 5.7 x 10^5 protoplasts g^{-1} fresh weight (Table 2).

In subsequent experiments, different explant material was tested. For callus induced on hairy roots, a comparable number of protoplasts as for callus induced on

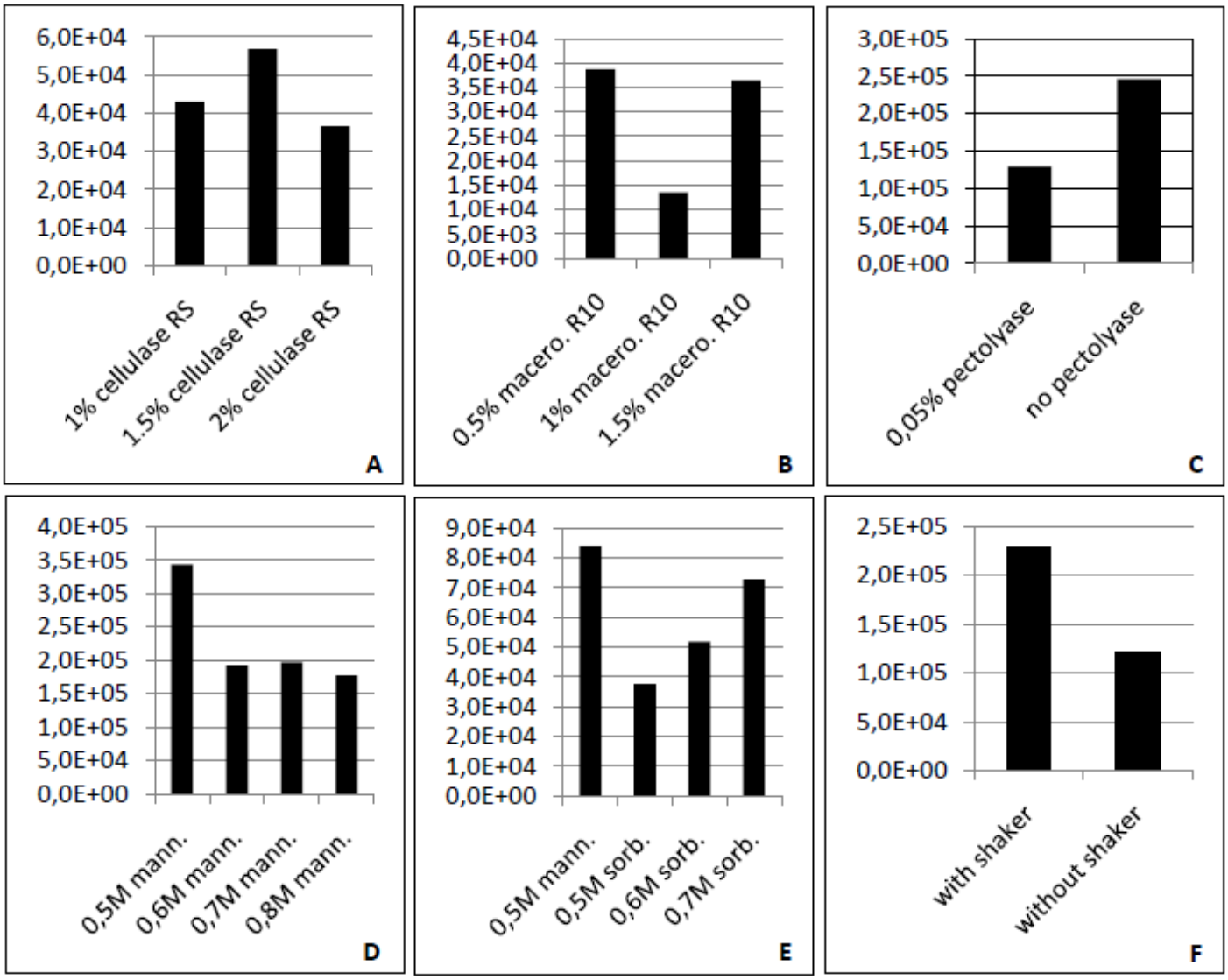

Figure 1. Preliminary tests to determine the optimal enzyme mixture. The basic mixture consisted of 2% cellulase RS, 0.5% macerozyme R-10 and 0.5M mannitol. In 6 independent tests (a) the cellulase concentration, (b) the macerozyme concentration, (c) the pectolyase concentration and (d) the mannitol concentration was varied. (e) Sorbitol was used as an osmoprotectant in different concentrations and (f) shaking during incubation in the enzyme mixture was beneficial.

Table 2. Comparison of different types of starting material for protoplast isolation and effect of incubation time on protoplast yield.

Starting material	Duration of incubation (h)	Number of protoplasts x 10^5 g^{-1} fresh weight ± SD	Mean diameter of the protoplasts (µm)
In vitro leaves	5 15	61 ± 1.9 52 ± 14	23 24
Callus from leaves	5 15	2.7 ± 1.0 5.7 ± 0.9	30 30
Callus from hairy roots	5 15	4.5 ± 0.9 3.2 ± 0.06	29 30
Hairy roots	5 15	8.8 ± 2.5 5.0 ± 1.8	14 15

Results are mean values of three independent repeats.

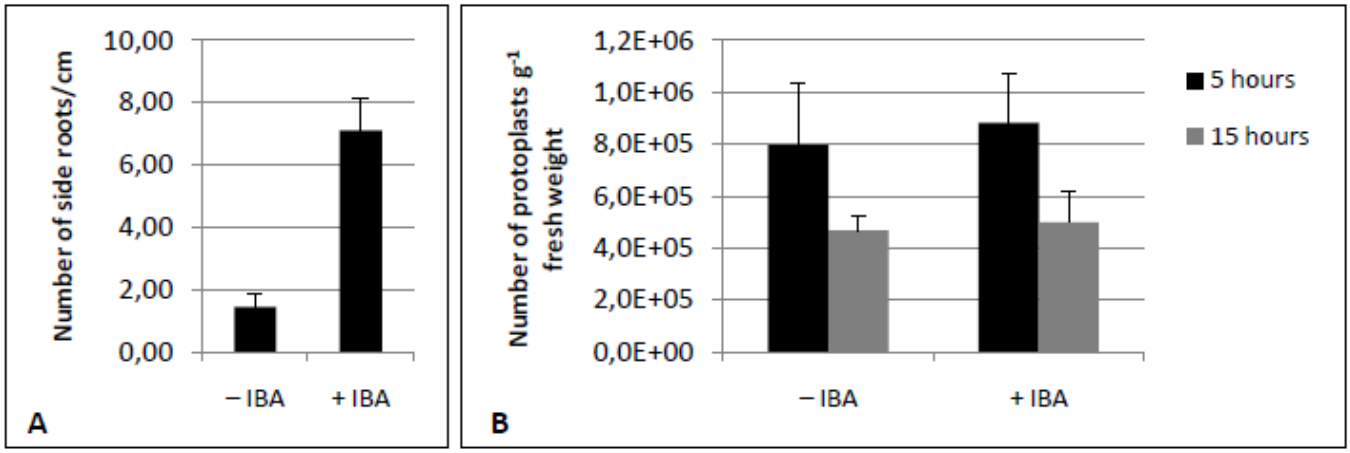

Figure 2. Effect of IBA treatment (a) on lateral root formation and (b) on protoplast yield, protoplast isolation tested for two incubation times, 5 and 15 hours. Results are mean values of three independent repeats.

leaves were obtained, namely 4.5 x 10^5 protoplasts g^{-1} fresh weight. Also for leaf material, the same protocol was highly efficient and a yield of 5 to 6 x 10^6 protoplasts g^{-1} fresh weight was obtained. In these experiments, there were no large differences in yield when protoplasts were isolated during 5 or 15 h (Table 2).

Protoplast isolation from transgenic hairy roots

The same enzyme mixture was tested directly with hairy roots. Preliminary tests showed that the yield was very low and not reproducible. Moreover, protoplasts derived from the differentiated parts of the hairy roots did not survive protoplast isolation very well (results not shown). In contrast, the root tips are composed of actively dividing cells which generated small protoplasts that persisted much better after the enzymatic treatment. Hence, only root tips were used to obtain stable protoplasts. Because the hairy root cultures contained limited numbers of root tips and their isolation was very labor intensive, we increased the number of root tips in the hairy root culture by applying lateral root inducing growth regulators. Addition of IBA strongly stimulated lateral root formation in *Maesa* hairy roots. Treatment of hairy roots with IBA resulted in 7 root tips cm^{-1} while control hairy roots that were on medium without growth regulator showed on average 1.5 side roots cm^{-1} (Figure 2a). Treatment with IBA clearly enhanced lateral root formation along the entire roots system, which turned the complete root good starting material for protoplast isolation.

Protoplast isolation from roots treated with IBA yielded slightly more protoplasts than non treated roots (Figure 2b). Control hairy roots produced 7.8 x 10^5 protoplasts g^{-1} fresh weight. Whereas IBA-treated roots produced 8.8 x 10^5 protoplasts g^{-1} fresh weight after 5 h of incubation in the enzyme solution. Incubation in the enzyme mixture for 15 h yielded less protoplasts with both treatments compared to 5 h incubation (Figure 2b). Although the difference in protoplast yield was not very different when treating roots with IBA or not, there was a significant difference in work load to obtain high protoplast numbers. Isolation of protoplasts treated with IBA was much more efficient and faster than isolation from non treated hairy roots.

Quality of the protoplasts

Viability of protoplasts directly after isolation was 90-98% with all types of starting material. However, morphology of protoplasts was clearly different depending on the type of explant used for protoplast isolation (Figure 3). Mean size for protoplasts of callus material was 30 µm, while the size of protoplasts from leaves was 23 -24 µm and of hairy roots only 14-15 µm (Table 2). Protoplasts from hairy roots and from callus induced on hairy roots also showed strong green fluorescence (Figure 3), which could be useful in further hybridization experiments to distinguish between fused and non-fused protoplasts.

DISCUSSION

M. lanceolata is a tropical plant, producing medically interesting saponins. Maesa saponins have strong anti-angiogenic action, which could make them useful for the treatment of certain types of tumors. It is, however, difficult to cultivate *M. lanceolata* in the greenhouse; for example, most of the plants do not flower. To circumvent out-door or greenhouse cultivation problems, we set up different types of *in vitro* cultures, namely shoot, callus and hairy root cultures. Currently, these cultures are being investigated for saponin production. Good

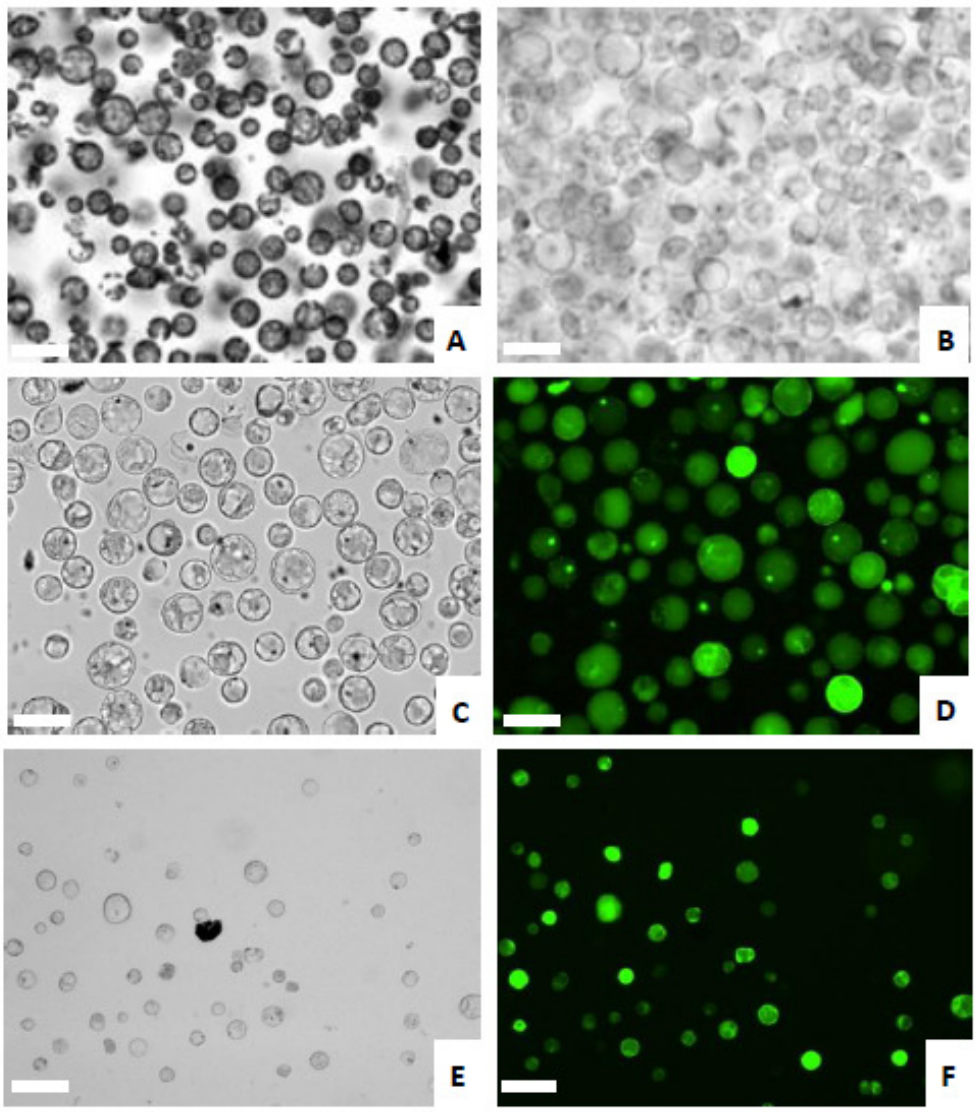

Figure 3. Protoplasts isolated from different types of tissue culture material. (a) Protoplasts from leaves of *in vitro* plants. (b) Protoplasts from callus induced on leaves. (c – d) Protoplasts from callus induced on transgenic hairy roots showing GFP fluorescence. (e – f) Protoplasts from transgenic hairy roots pretreated with IBA, also with GFP fluorescence. Scale bars are 50 µm.

producing lines will be upscaled and grown for saponin identification and quantification. Tissue or cell culture material can indeed be a useful tool for the production and characterization of pharmaceutical valuable secondary metabolites (Vanisree et al., 2004). For example, *in vitro* root cultures of *Catharanthus roseus* are used for the commercial 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 the alkaloid taxol. Taxol 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). Also ginseng saponins, which have been recognized as promoters of health and longevity and are the major constituents of many health drinks and teas, are produced through *P. ginseng* tissue cultures. Large-scale suspension culture of ginseng cells was already reported in 1972 by Yasuda and coworkers (Yasuda et al., 1972). Later on, in the 1980s, an industrial-scale culture process was initiated by Nitto Denko Corporation (Ibaraki, Osaka, Japan) using stirred tank fermentors to achieve productivities of 500 - 700 mg l^{-1} day^{-1}.

In this study a method for isolation of protoplasts from leaves, callus and hairy roots was developed and

optimized. For all three types of starting material the same enzyme mixture (1.5% cellulase, 0.5% macerozyme and 0.5 M mannitol) was highly effective. The highest yield of protoplasts was 6.1 x 10^6 g^{-1} fresh weight when starting from leaf material. Callus yielded at maximum 5.7 x 10^5 protoplasts g^{-1} fresh weight. These protoplast yields are better or comparable to the results described in literature. For example, for *Calibrachoa* leaves 1.1 – 4.6 x 10^6 protoplasts g^{-1} fresh weight and for *Petunia* 1.0 – 3.6 x 10^6 protoplasts g^{-1} fresh weight was achieved (Meyer et al., 2009). Leaves of *Spathiphyllum* and *Anthurium* yielded respectively 9 x 10^5 and 7 x 10^4 protoplasts g^{-1} fresh weight (Duquenne et al., 2007). For callus of *Echinacea* 5.0 x 10^5 and for callus of *Agapanthus* 0.8 – 1.5 x 10^5 protoplasts g^{-1} fresh weight was obtained (Nakano et al., 2003; Zhu et al., 2005).

Very few studies report on the isolation of protoplasts from hairy roots (Sevon et al., 1997, 1998). Yet, hairy roots are good source material for the production of transgenic protoplasts expressing marker genes like GFP which could be helpful for protoplast fusion experiments and tracking the regeneration process. The isolation of protoplasts from hairy root cultures does not produce sufficiently high yield for protoplast fusion or transformation experiments. This is probably due to the relatively low representation of meristematic cells with digestible cell walls. To obtain a good protoplasts yield, root tips must be separated and collected for enzyme treatment. The isolation of the roots tips is very difficult and laborious and requires the growth of large quantities of hairy root cultures. By means of application of the lateral root inducing hormone IBA, we were able to increase the number of root tips, allowing high yield protoplast isolation from hairy root cultures directly, without isolating root tips. Although plants contain less IBA than IAA *in vivo*, IBA more efficiently induces lateral root formation, hence its use in agricultural applications (De Klerk et al., 1999; Hartmann et al., 1990). Treatment of hairy roots with IBA resulted in 5 times more lateral roots cm^{-1} compared to non treated controls. Isolation of protoplasts from treated and non treated hairy roots yielded 8.8 x 10^5 and 8.0 x 10^5 protoplasts g^{-1} fresh weight, respectively.

Although that the yield of protoplast was similar, the process of protoplast isolation was greatly facilitated when hairy roots were treated with IBA. Further studies will focus on applications of protoplast technology. Previously, we have tried to transform *M. lanceolata* with *Agrobacterium tumefaciens* to obtain transgenic shoots. However, *Maesa* proves to be very difficult to transform (unpublished results). Transformation of protoplasts has been successful in a number of plant species (Davey et al., 2005; Krasnyanski et al., 1999; Krens et al., 1996; Winfield et al., 2001) and DNA uptake into protoplast has been especially important in transforming plants that are not amenable to other methods of gene delivery, specifically *Agrobacterium* mediated methods (Davey et al., 2005; Rakoczy-Trojanowska, 2002).

In addition, plant protoplast fusion is an important technology for the generation of novel plant varieties and hybrids (Davey et al., 2005). For example, the limitations of crossing related plant species with agriculturally important traits due to sexual incompatibility can be overcome by somatic hybrids using protoplasts. In bacteria and fungi the technique of protoplast fusion is also used extensively to produce hybrid microorganisms with altered secondary metabolite characteristics. In plants, however, there are few examples of this application of protoplast fusion. Laurila et al. (1996) showed that protoplast fusion between *S. brevidens* and *S. tuberosum* can lead to the production of a novel secondary metabolite that is not present in the parental species (Laurila et al., 1996). Through hybridization, an enzyme with a specific activity is transferred from one species to the other species, with a different potential substrate, thus generating opportunities for novel biosynthetic reactions. Similar results were published recently by Saverese and co-workers when analysing the glycoalkaloid content of somatic hybrids of *Solanum tuberosum* and *S. bulbocastanum* (Savarese et al., 2009).

For the first time, a high yielding protoplasts isolation method was developed for the medicinal plant *M. lanceolata*. The method was successful for different tissue culture material as well as for hairy root cultures, allowing the isolation of transgenic protoplasts. These protoplasts will facilitate further research on secondary metabolism and the biosynthesis of saponins in *Maesa* species.

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