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Studies on saponin production in tropical medicinal plants *Maesa argentea* and *Maesa lanceolata*

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Abstract. The continuous need for new compounds with important medicinal activities has lead to the identification and characterization of various plant-derived natural products. As a part of this program, we studied the saponin production from two tropical medicinal plants *Maesa argentea* and *M. lanceolata* and evaluated several treatments to enhance their saponin production. In this experiment, we present the analyses of saponin production from greenhouse grown plants by means of TLC and HPLC-MS. We observed that the content of saponin from these plants varied depending on organ and physiological age of the plants. In addition, the impact of elicitors on saponin accumulation on *in vitro* grown plants was analyzed using TLC. The production of saponin was very stable and not affected by treatment with methyl jasmonate, and salicylic acid. In conclusion, *Maesa* saponins are constitutively produced in plants and the level of these compounds in plants is mainly affected by the developmental or physiological stage.

INTRODUCTION

The first report on triterpenoid saponins in *Maesa lanceolata* and *M. argentea* was published by Sindambiwe and co-workers within the framework of research on natural anti-infectious agents [1, 2]. The chromatographic and spectroscopic investigation on *M. lanceolata* saponin fractions resulted in the isolation and identification of 10 acylated triterpenoid saponins and were named maesasaponins [2]. Subsequently, some of the saponins in *M. argentea* were confirmed through comparison of the molecular weight, MS fragmentation pattern and retention time with those of reference samples of maesasaponins.

These two species play an important role in traditional medicine and ancient practices, whereby extracts of leaves and fruits are used for the treatment of various diseases including hepatitis, dysentery, skin diseases and neuropathies [1]. *In vitro* bioactivity assays showed that *M. argentea* and *M. lanceolata* have virucidal, molluscicidal, cytotoxic, haemolytic and anti-leishmanial actions [1, 3].

Currently, the medicinal uses of *Maesa* are based on collecting from natural resources. It means that the supply of raw material depends on seasonal availability, species abundance, plant growth rate and other uncontrolled environmental conditions. In addition, high-value secondary metabolites are often found in low abundance in nature, thus unreliable to fulfill the needs of pharmaceutical industry. To preserve global biodiversity and alleviate problems associated with field production, plants can be cultivated either through conventional cultivation or via plant cell and tissue cultures.

The *in vitro* culture and propagation method for *Maesa* species were successfully established [4]. Using in vitro grown material, we studied the saponin production in more detail and in addition to determined saponin production in greenhouse grown plants. Furthermore, the effect of factors possibly influencing saponin content, including organ type, age, phytohormone and elicitor treatments was also investigated.

MATERIAL AND METHODS

Plant Material

M. lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). The in vitro plants were maintained as previously described in Faizal (2011) [4].

Hormone Treatment of *In Vitro* Shoots

ABA, 2,4-D, GA3 and SA were tested at 0.01 mM and were dissolved in water. MeJA was dissolved in 100 % ethanol and used at 0.1 mM. Plantlets were removed from the culture medium in the tissue culture containers and were submerged in the phytohormones or control solutions for 30 seconds. Afterwards, every plant was placed into a

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glass tube containing solid MS medium supplemented with MS vitamins, 3% (w/v) sucrose and 0.8% (w/v) agar (Lab M plant tissue culture agar MC29, Amersham, UK). The cultures were placed in a growth room with 16/8 h light/dark conditions at 26 °C. Samples (1st – 6th leaves, starting from the apex) were harvested for saponin extraction 48 h after treatment. Saponin content was investigated with Thin Layer Chromatography (TLC).

Saponin Analysis

Thin Layer Chromatography

For TLC analysis, plant material was ground with liquid nitrogen. 250 µL of 50% (v/v) methanol was added and samples were sonicated twice for each 1 h and the extract were combined. 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 (F254) (Merck KGaA, Germany). For the mobile phase, the upper layer of a mixed of n-butanol/acetic acid/H2O was used. 10 µL of the samples was spotted at 2 cm from the sides and bottom of the plate. The saponins were stained using an anisaldehyde reagent (1 mL/L p-anisaldehyde, 20 mL/L acetic acid, 170 mL/L methanol and 10 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.

RESULT

Saponin Production in *Maesa* Greenhouse Grown Plants

Saponin Content in Different Organs

To analyze the presence of saponins in different organs, crude extracts were made from leaves, stems and roots of 1 month old *M. argentea* and *M. lanceolata*. These were subjected to thin layer chromatography (Fig. 1).

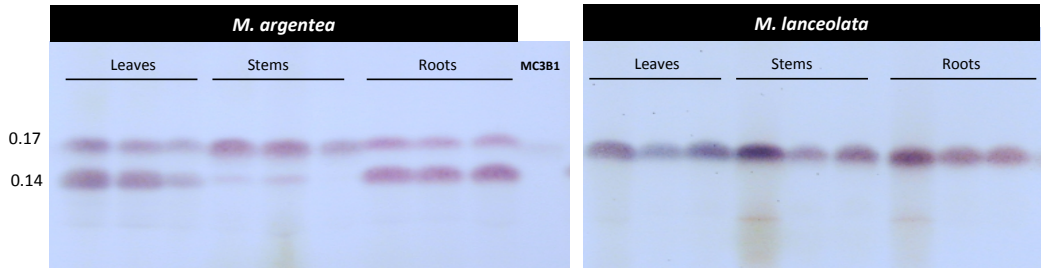

FIGURE 1. TLC analysis from different organs of *M. argentea* and *M. lanceolata.* For each species, three independent plants were used for extraction. An HPLC purified maesasaponin mix (0.1% MC3B1) was used as reference sample.

Using purified *M. lanceolata* saponin mixture (MC3B1) as a reference standard, qualitative TLC analysis showed that saponin from *M. lanceolata* was characterized by the appearance of single spot at R_f= 0.17. *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. [3]. On the other hand, a spot at R_f = 0.14 being the most abundant in leaves and roots suggests that *M. argentea* produces saponins quite distinct from the maesasaponins described for *M. lanceolata.*

Saponin Content in Maesa Plants with Different Ages

Maesa species tested in the previous experiments are from 3-month-old plants. However, seedlings or juvenile plants can be physiologically quite different from adult plants. To investigate whether saponin content varies with age, we analyzed saponin content from leaves of greenhouse grown plants from approximately 1 month, 9 months and 2 years old. TLC results are shown in Fig. 2.

For *M. argentea* two characteristic bands, with R_f 0.14 and 0.17, were obtained. For both bands, the intensity and surface area increased as the age of the plants increased. *M. lanceolata* had one band with R_f 0.17 and, although there was some variation between different plants of the same age, it was clear that leaves of 2 year old plants had a higher

saponin content than leaves of the younger plants. In general, we conclude that saponin production in leaves of *Maesa* plants markedly increased with increasing age of the plants.

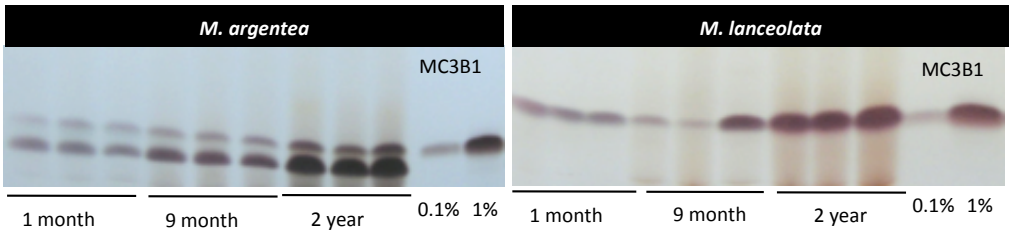

FIGURE 2. TLC analysis of saponin extracts from leaves of *M. argentea*, *M. balansae*, *M. lanceolata* and *M. perlarius* plants growing in the greenhouse for 1 month, 9 months and 2 years. An HPLC purified maesasaponin mix (MC3B1) was used as reference sample. The three repeats correspond to extracts from individual plants.

Saponin Production in In Vitro Cultures

The Influence of Phytohormones on In Vitro Culture

Phytohormones play important roles in regulating developmental processes and signaling networks involved in plant responses to a wide range of biotic and abiotic stresses. They are also very often used in tissue culture techniques. Therefore, plant hormones are a potential source of variation in terms of secondary metabolite accumulation in in vitro plant cultures. Several reports have described the upregulation of secondary metabolites upon treatment with phytohormones [5]. Therefore, it would be interesting to see if plant hormones such as gibberellic acid (GA3), 2,4-dichlorophenoxyacetic acid (2,4-D), and abscisic acid (ABA) can influence saponin biosynthesis in *Maesa*.

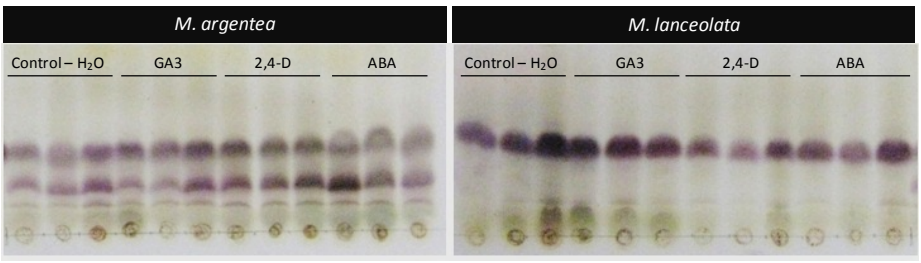

FIGURE 3. TLC of in vitro plantlets of *M. argentea* and *M. lanceolata* upon exposure with different hormones; 0.01 mM gibberellic acid (GA3), 0.01 mM 2,4-dichlorofenoxyacetic acid (2,4-D) and 0.01 mM abscisic acid (ABA). Controls were treated with water and all samples were harvested 48 h after treatment. Different repeats represent separate plants.

None of the plants showed major morphological changes after treatment with any of the phytohormones within the time of analysis (Fig. 3). Therefore, we assume that short hormone treatments do not strongly influence metabolite sinks. Treatment with GA3, 2,4-D and ABA did not lead to major changes in saponin content as determined by TLC. Although there were differences between the controls and the treated samples, these were most probably due to the extraction and/or TLC procedure. To address the problem of inadvertent technical mistakes, each treatment was analyzed by extracting saponins from three individual plants separately. The variations occurring in samples from treated plants were also noticed in control extracts.

In addition to the phytohormones, we also tested the effect of methyl jasmonate (MeJA) known as a universal elicitor. Jasmonates are derived from the metabolism of membrane fatty acids, which are widespread in the plant kingdom. These compounds are involved in crucial processes related to plant development and survival, including, senescence, reproduction, fruit development, direct and indirect defense responses, and secondary metabolism [6, 7]. Among jasmonates, jasmonic acid (JA) and methyl jasmonate (MeJA) are commonly used for enhancing secondary metabolites production like saponins [8]. Therefore, we tested the effect of 0.1 mM MeJA on in vitro shoots of all four *Maesa* species and investigated saponin production with TLC (Fig. 4). Plantlets were dipped in a solution for 30 sec and then placed on fresh medium.

TLC analyses showed that there were no increases in saponin concentration of four *Maesa* species. These results are in contrast with MeJA effects on other secondary metabolites which often increase upon MeJA treatment [9]. This demonstrated that *Maesa* saponins are produced in a stable fashion and not affected by MeJA treatment.

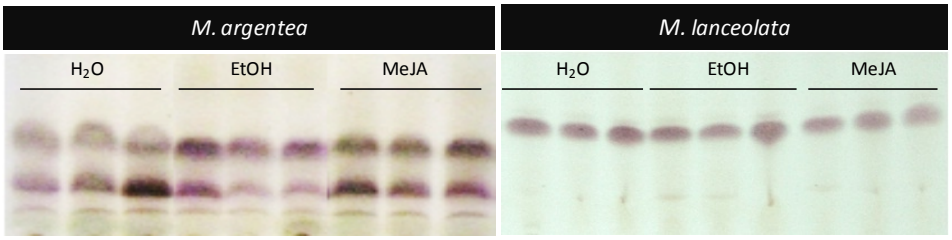

FIGURE 4. TLC of in vitro plantlets of after exposure with 0.1 mM methyl jasmonate (MeJA). Control leaves were treated with H_2O and with ethanol. Samples were harvested 48 h after treatment. Different repeats represent different plants

DISCUSSION

TLC analyses of *Maesa* greenhouse grown plants revealed that saponins were present in leaves, stems and roots of *M. argentea* and *M. lanceolata*, although, there were considerable qualitative and quantitative variations observed between different organs. These differences between organs have also been reported in other species such as *Medicago trucantula* and *Avena* spp. (oats). Conjugates of the triterpene saponin medicagenic acid were highly accumulated in leaves of *M. trucantula*, while soyasapogenol was higher in the root [10]. In oats, the distribution of the two groups of saponins produced is mutually exclusive since avenacosides (steroidal saponins) are produced in the leaves and avenacins (triterpenoid saponins) in the roots [11]. The differential distribution of specific saponins is most likely the result of spatially controlled biosynthesis, specific function, and active transport.

In addition to differences in saponin content in different organs, we also observed differences in saponin concentration depending on the developmental stage. Older plants produced more saponins compared with younger plants. This confirms that plant age is one of the important factors influencing saponin content as has been reported in many plant species. In *Medicago sativa*, the concentration of medicagenic acid and zanhic acid respectively increased by nearly 1.5 and 2 fold passing from the second to the third year of growth [12]. The age of harvested plants is also essential for all species of ginseng. The amount of ginsenosides increased from 1 to 5 year old roots of *P. ginseng* and *P. quinquefolius* [13].

As the most studied species for in vitro analysis of saponins, *P. ginseng* has also been studied for phytohormone enhancement of saponin production. Treatment of *P. ginseng* adventitious root with 0.025 mM indole-3-butyric acid (IBA) increased saponin content 1.6 fold [14]. In *P. quinquefolium* cell cultures, the content of saponins varied depending on the hormones in the culture medium [15]. However, in this study short treatment of *Maesa* in vitro shoots with three different types of hormones; GA3, ABA and the auxin 2,4-D had no effect on saponin production.

We have also shown that saponin content was higher in more mature leaves and older plants. Therefore, it may be suggested that auxin and/or gibberellic acid are responsible for the increase in saponin production since these growth regulators are generally involved in leaf growth. However, a short contact of in vitro *Maesa* leaves with auxin or gibberellic acid did not significantly change saponin production. Consequently, the increased saponin production in mature *Maesa* leaves cannot be a direct effect of the higher auxin and/or gibberellic acid levels in leaves.

Another important method to enhance saponin production is via treatment with elicitors [16]. The elicitation process is generally regarded as the expression of defense-related genes and activating defense-related secondary metabolic pathways. Elicitation is a very complex process and depends on many factors such as elicitor concentration, growth stage of the culture at the time of elicitor addition and contact time with the elicitor [17]. In addition, the response to a particular elicitor may vary from plant to plant. In this context, we studied the effect of MeJA on saponin accumulation from two *Maesa* species. The addition of JA and/or MeJA has been reported to strongly enhance production of ginsenoside, saponin in adventitious root cultures of *P. ginseng* [18] and the accumulation of saponin in whole plant culture of *Centella asiatica* [19]. In contrast, none of these treatments show significant increase in saponin production from *Maesa*. Therefore, we conclude that *Maesa* saponins are constitutively present in plants. The level of these compounds is significantly influenced by intrinsic factors reflecting the physiological status of the plants, in particular the developmental stage and age of the plant. In addition, they are produced in a stable manner irrespective of external factors or environmental stimuli.

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