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EFFECTS OF SEMI-SOLID AND LIQUID MEDIA ON THE DEVELOPMENT OF *SMILAX MYOSOTIFLORA* PLANTLETS

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This study aims to establish an efficient tissue culture protocol for *Smilax myosotiflora* (ubi jaga) by comparing the effects of semi-solid and liquid (temporary immersion system, TIS) media on shoot multiplication, root induction, and acclimatisation. Semi-solid and liquid media were tested for complete plantlet regeneration. In semi-solid media, 2.0 mg/L BAP induced 67% shoot multiplication with an average of one shoot per explant. In liquid media, over 80% of shoots multiplied, averaging 3.5 shoots per explant at the same BAP concentration. For root induction, half-strength MS semi-solid media with 0.5 mg/L IBA produced 60% rooting, while liquid media yielded only 12% under identical conditions. Acclimatisation in Jiffy 7® showed shoots from liquid media had over 90% survival compared to 52% from semi-solid after 3 weeks. Chemical analysis of leaf extracts from *in vitro* and one-year-old *ex vitro* plants detected only low levels of possible caffeic acid derivatives. This is the first optimised *in vitro* protocol for *S. myosotiflora* using TIS, offering strong potential for large-scale commercial propagation and serving as a valuable resource for future germplasm conservation initiatives. Overall, the tissue culture protocol used for *S. myosotiflora* has been successfully established.

Keywords: Micropropagation, temporary immersion system (TIS), ubi jaga, bioreactor, tissue culture

INTRODUCTION

Smilax myosotiflora, locally known as ubi jaga in Malaysia, is an herbaceous climber with a slender, smooth stem from the Smilacaceae family. This species is distributed in Peninsular Malaysia, Southern Thailand, and Indonesia, typically occurring in lowlands and foothills (Rosdi et al. 2022). Traditionally, *S. myosotiflora* has been used to treat syphilis, rheumatism, and various skin ailments, including wounds, inflammations, and ulcers (Ong & Azliza 2015). Rhizome or tuber decoctions are also consumed as male tonics and have been reported to possess antibacterial, antifungal, antioxidant, and immunomodulatory activities (Mustaffar Bakri 2013, Wan et al. 2016, Chyang et al. 2018). Due to these reported medicinal properties, particularly antioxidant and phytoestrogenic potential, the species has attracted growing interest from herbal and pharmaceutical industries.

Wild harvesting remains the primary source of *S. myosotiflora*, yet information on conventional propagation methods is scarce and poorly documented. As with other *Smilax* species, propagation is presumed to occur via seeds, stem cuttings, and rhizomes (Dong et al. 2014), but harvesting rhizomes requires specialised techniques. Limited propagation, high market value, and overharvesting pressures have increased the species' vulnerability, making it a priority for both conservation and large-scale propagation initiatives.

Plant tissue culture offers an effective means of mass propagating rare and commercially valuable species such as *S. myosotiflora*. Using nodal segments or shoots, large quantities of plants can be produced under controlled environmental and nutritional conditions. In this study, clean cultures were derived from nodal segments of a DNA-authenticated mother plant, identified morphologically by a botanist and confirmed through DNA barcoding at the Forest Research Institute Malaysia (FRIM), following methods described by Tnah et al. (2015). Two culture approaches—semi-solid medium and a temporary immersion system (TIS)—were compared for their suitability in multiplication and growth. TIS is a liquid-culture bioreactor that intermittently immerses explants in nutrient media, improving aeration, nutrient uptake, and scalability (Uma et al. 2021).

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Previous studies have shown that *S. myosotiflora* responds well to Murashige and Skoog (MS) medium with 6-benzylaminopurine (BAP). Shoot regeneration has been achieved on semi-solid medium (Rasyidah et al. 2016), while static liquid culture has also been tested (Hafawati et al. 2023). However, semi-solid culture generally outperformed static liquid systems, and no study has evaluated the use of TIS for this species. In addition, the influence of culture system on post-acclimatisation survival and the *in vitro* production of potential bioactive compounds in *S. myosotiflora* has not been explored.

This study was therefore undertaken to establish an efficient *in vitro* propagation protocol for *S. myosotiflora* using TIS and to compare its performance with semi-solid culture in terms of shoot multiplication, root induction, and acclimatisation. A further aim was to conduct a preliminary biochemical screening of *in vitro* and *ex vitro* leaves for possible caffeic acid derivatives, providing baseline information for future phytochemical enhancement and commercial exploitation.

MATERIALS AND METHODS

In vitro shoot multiplication in two different medium types

Two different media types were used in this study; semi-solid and liquid media. Shoot multiplication experiments for *S. myosotiflora* were first conducted using semi-solid media, which served as a baseline for subsequent scale-up using a temporary immersion system (TIS). Murashige and Skoog, MS (Murashige & Skoog 1962) basal medium supplemented with 0, 0.25, 0.5, 1.0, and 2.0 mg/L 6-benzylaminopurine (BAP) were prepared with 3% (w/v) sucrose and 0.3% (w/v) gelrite (Duchefa Biochemie, G1101) as solidifying agents, and pH was set at 5.8 prior to autoclaving. Three shoots with approximately 3–4 cm in length were cultured in each medium bottle, and each treatment was repeated three times. All cultures were kept in a growth room with a parameter of 2000 lux, 16/8-hour light, and a temperature of 22 ± 2°C. The final observation was made after 8 weeks of culture, when the number of new shoots and shoot height were recorded.

The temporary immersion system, brand RITA®, was used in this study, and *S. myosotiflora* shoot cultures from semi-solid media were used as explants. BAP with concentrations of 0.5 to 2.0 mg/L was used singly or in combination with 0.5 mg/L indole-butyric acid (IBA) to induce shoot multiplication. The immersion frequency and duration were set to six times per day for five minutes. As a control, MS basal media without the addition of a solidifying agent were used. The culture vessels were filled with 250 mL of liquid MS basal media with the respective PGR and set at pH 5.8 prior to autoclaving. Ten shoots of 3–4 cm in length with at least two nodes were cultured and repeated three times. The explants were laid horizontally in the TIS vessels. All cultures were kept in a growth room with a parameter of 2000 lux, 16/8-hour light, and a temperature of 22 ± 2 °C. The final observation was made after 8 weeks, when the number of new shoots and shoot height were recorded.

In vitro root induction

Experiments on *S. myosotiflora* root induction were done using semi-solid and liquid media. For semi-solid, half-strength MS basal supplemented with a range of auxin (NAA and IBA) concentrations from 0, 0.5, 1.0, and 3.0 mg/L were tested. The experimental media were prepared with supplementation of 3% (w/v) sucrose and 0.3% (w/v) gelrite as solidifying agents, and the pH was set at 5.8 prior to autoclaving. Each medium was cultured with 3 shoots of 4–5 cm height and repeated three times. The culture experiment was kept in a growth room with a parameter of 2000 lux, 16/8-hour light, and a temperature of 22 ± 2 °C. The final observation was made after 4 weeks, when the number of new shoots, shoot height, and root emergence were recorded.

For liquid media, TIS vessels were prepared with rooting induction medium experiments with three replicates each. Different basal MS strengths (¼ MS, ½ MS, ¾ MS, and full MS) with the addition of 0.5 mg/L IBA were prepared. The culture vessels were filled with 250 mL of liquid media and set at pH 5.8 prior to autoclaving. Ten shoots with 4–5 cm were cultured in the vessel and repeated three times. The duration and frequency of the immersion were set to six times per day for five minutes. The culture experiment was kept in a growth room with a parameter of 2000 lux, 16/8-hour light, and a temperature

of 22 ± 2 °C. After 4 weeks of culture, the final observation was made, and root numbers and root length were recorded.

Acclimatisation

Rooted and non-rooted plantlets produced from both semi-solid and liquid media were acclimatised in the weaning chamber for 3 weeks. Non-rooted plantlets were treated with a commercial rooting hormone, Seradix® No. 3, by dipping the shoot base into the powder before transplanting into Jiffy 7® potting mix. Seradix® No. 3 is commonly formulated with approximately 0.8% (w/w) indole-3-butyric acid (IBA) and is typically recommended for semi-hardwood cuttings. Jiffy 7®, which contained sphagnum peat moss, was used as a medium during the acclimatisation process. The plantlets were washed under running tap water to remove any traces of media agar. The plantlets were treated with the fungicide Thiram 80WP at a concentration of 0.1% (w/v) and subsequently transferred into Jiffy 7® peat pellets prior to the acclimatisation process. The survival rates of the plantlets were recorded after 3 weeks. The surviving plantlets were transplanted into a soil mixture of baked soil and peat moss (2:1) and grew under shade in the nursery.

Leave extract preparation

For both the semi-solid and liquid culture treatments, leaves were collected from *S. myosotiflora* plantlets at the shoot multiplication stage, after three months of *in vitro* culture. Only leaves longer than 3 cm were selected. To ensure representative sampling within each culture system, shoots were obtained from multiple culture bottles (semi-solid) and multiple TIS vessels (liquid). For nursery-grown plants, leaves were sampled approximately one year after acclimatisation and transfer into polybags. In each treatment group, leaves from multiple plantlets were pooled to form one biological replicate. Leaves were oven-dried at 40 °C for approximately 3 to 5 days, or until they became completely dry and brittle, to ensure thorough moisture removal and preserve phytochemicals prior to extraction. The dried leaves were then ground into powder form. The leaf samples were filtered with a 500 µm filter prior to the extraction process. Methanol was added to the sample at a ratio of 0.5 g/5.0 mL and sonicated for 15 min to facilitate cell wall destruction and compound release. The mixture was later filtered with a 0.45 µm PTFE membrane filter prior to analysis by high-performance liquid chromatography (HPLC).

High Performance Liquid Chromatography (HPLC) analyses

HPLC (Waters 2535 quaternary gradient pump, Waters 2707 auto sampler, and Waters 2996 PDA) and HPLC Phenomenex Luna C18 (2), 5 µm, 250 mm × 4.6 mm) column with solvent system consisting of A (0.1% formic acid in water), B (acetonitrile), and C (methanol) were used to quantify caffeic acid in *S. myosotiflora* extract. The solvent system setting was as shown in Table 1.

Table 1 The flow rate and solvent system for the HPLC analysis

Time (min)	% A (0.1% formic acid in water)	% B Acetonitrile	% C (Methanol)
0	90	10	0
50	60	30	10
55	60	30	10
60	90	10	0
62	90	10	0

Note: The flow rate was set at 1 mL/min with a sample volume of 10 µL.

Data analysis

Tukey's honestly significant difference (HSD) test ($p = 0.05$) was performed when significant differences among treatments were detected by analysis of variance (ANOVA) on the shoot multiplication and root induction data. The analyses were carried out using SAS 9.1.2.

RESULTS AND DISCUSSION

Complete plantlets regeneration of *S. myosotiflora* shoot multiplication

S. myosotiflora responded slowly in semi-solid culture and required higher BAP concentrations for shoot induction. BAP at 0.25 and 0.5 mg/L concentrations was unable to induce new shoot formation in *S. myosotiflora*. This lack of response

may be attributed to insufficient cytokinin levels to stimulate the necessary cell division and meristematic activity required for shoot initiation. Cytokinins like BAP are known to promote cell proliferation and shoot organogenesis, but their effectiveness is highly concentration-dependent and varies by species and explant type (Nazirah et al. 2021, Oliveira et al. 2017). In the case of *S. myosotiflora*, these lower concentrations were likely below the physiological threshold for shoot bud differentiation. Higher BAP concentrations (1.0 mg/L and 2.0 mg/L) were finally able to induce a new shoot, but the mean number of new shoots induced per explant was only 0.833, even though the percentage frequencies were 50% and 70%, respectively (Table 2, Figure 1). Observation indicated the bases of *S. myosotiflora* shoot cultures have shown the exudation of phenolic compounds, a brown substance that leaches into the culture medium. There were shoot tip necrosis (STN) events observed in a few cultures. The shoots survive; however, this event probably affects their capability to multiply and grow. The observed browning and necrosis highlight a clear limitation of the present protocol. Therefore, future research should investigate the efficacy of anti-browning additives—such as 2-aminoindane-2-phosphonic acid (AIP), polyvinylpyrrolidone (PVP), activated charcoal, and antioxidants—that have been successfully used in other species to enhance shoot proliferation and quality in *S. myosotiflora* cultures (Jones & Saxena 2013, Amente & Chimdessa 2021).

In *S. corbularia* and *S. zeylanica*, shoot multiplication per explant was also low, with maximum means of 1 and 2 shoots, respectively. *S. corbularia* showed a higher percentage of bud break (95%–100%) than the present study but still produced only one shoot per explant

Table 2 *Smilax myosotiflora* response in MS semi-solid media supplemented with BAP at different concentrations after 8 weeks in culture for shoot multiplication

PGR concentration (mg/L) in MS semi-solid media	Percentage of explant producing new shoot (%)	Mean number of new shoot ± SE	Mean height of new shoot (cm) ± SE
0 mg/L BAP	0	0 ± 0^{b}	0 ± 0^{a}
0.25 mg/L BAP	17	0.833 ± 0.307^{a}	0.916 ± 0.304^{a}
0.5 mg/L BAP	33	0.333 ± 0.167^{ab}	0.333 ± 0.167^{a}
1.0 mg/L BAP	50	0.333 ± 0.167^{ab}	0.422 ± 0.217^{a}
2.0 mg/L BAP	67	0.556 ± 0.242^{ab}	0.756 ± 0.345^{a}

Note: Means with different letters in each column are significantly different (Tukey's HSD test), $p \leq 0.05$. SE = standard error.

Figure 1 Tissue culture-derived *Smilax myosotiflora* plantlets in semi-solid media

Note: The figures show the shoot multiplication of *Smilax myosotiflora* in semi-solid media (left) and rooted *Smilax myosotiflora* in root induction semi-solid media (right)

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when cultured on MS medium with 15% (v/v) coconut water and 1 mg/L BA, suggesting higher cytokinin levels were needed for multiplication (Jirakiattikul et al. 2013). In *S. zeylanica*, 60% bud break occurred on half-strength semi-solid MS supplemented with auxin, 2 mg/L kinetin, and activated charcoal (Thirugnanasampandan et al. 2009). Across *S. myosotiflora, S. zeylanica*, and *S. corbularia*, multiplication rates were slow despite high bud break. In *S. myosotiflora*, full-strength semi-solid MS induced high bud break, but greater BAP concentrations or combinations with auxins such as NAA or IBA may be needed to enhance multiplication, as cytokinins mainly initiate bud break while auxins can promote elongation and proliferation.

To our knowledge, this is the first report on the use of a temporary immersion system (TIS) for the *in vitro* shoot induction and multiplication of *S. myosotiflora*. In this study, MS liquid medium supplemented with BAP at different concentrations, either applied alone or in combination with 0.5 mg/L IBA, was used to evaluate shoot development under TIS conditions. While previous studies have reported the use of static liquid or semi-solid cultures, the application of TIS represents a novel approach for improving propagation efficiency in this species. Table 3 shows the response of *S. myosotiflora* after 8 weeks in culture. In this study, the highest mean number of new shoots was 3, and the percentage frequency was 87% in MS basal supplemented with 2.0 mg/L BAP. These two figures are relatively higher than the number of shoot inductions and percentage frequency of *S. myosotiflora* (Table 2), *S. corbularia* and *S. zeylanica* in semi-solid media (Thirugnanasampandan et al. 2009, Jirakiattikul et al. 2013). Even though the concentration of BAP used is similar (2.0 mg/L), shoot induction in semi-solid media is lower than in TIS. The use of TIS increases the exposure of each node on the explants to the media. The explants were laid horizontally in the vessel, and the whole plant surface was directly exposed to the nutrients and hormones. In semi-solid media, only the basal part was exposed to the media and the nutrients and hormones were transported from the basal part to the shoot tip. The combination of hormones (BAP and IBA) has no discernible effect on shoot multiplication. Therefore, 2.0 mg/L BAP without IBA is the appropriate medium for *S. myosotiflora* shoot induction in TIS. Figure 2A shows the shoot multiplication of *S. myosotiflora* in TIS.

According to Teixeira et al. (2020), the *S. zeylanica* shoot culture suffered from shoot tip necrosis (STN), which was caused by the exudation of a phenolic compound. *S. myosotiflora* was also noted to suffer from shoot tip necrosis in the semi-solid medium, and the medium colour changed from clear white to light brown. This situation signifies the exudation of the phenolic compound. In the TIB system, the media colour changes were also noted, but no shoot tip necrosis was observed. Temporary immersion, as the term implies, refers to periodic immersion in the medium, and it most likely lessened the excess phenolic effect on the explant cell.

Besides phenolic compound exudation, other factors that cause STN are nutrient deficiency, high relative humidity, and high ethylene production due to inadequate ventilation

Table 3 *Smilax myosotiflora* response in MS liquid media supplemented with different PGR concentrations after 8 weeks in culture for shoot multiplication in TIS

PGR concentration (mg/L) in MS liquid media	Percentage of explant producing new shoot (%)	Mean number of new shoot ± SE	Mean height of new shoot (cm) ± SE
0.5 mg/L BAP	87	2.60 ± 0.32^{ab}	1.53 ± 0.19^{ab}
0.5 mg/L BAP + 0.5 mg/L IBA	47	0.53 ± 0.12^{c}	0.85 ± 0.20^{b}
1.0 mg/L BAP	60	1.77 ± 0.38^{bc}	1.32 ± 0.26^{ab}
1.0 mg/L BAP + 0.5 mg/L IBA	80	2.17 ± 0.37^{ab}	1.55 ± 0.23^{ab}
2.0 mg/L BAP	87	3.47 ± 0.37^{a}	1.84 ± 0.17^{a}
2.0 mg/L BAP + 0.5 mg/L IBA	60	1.40 ± 0.30^{bc}	0.93 ± 0.23^{b}

Note: Means with different letters in each column are significantly different (Tukey's HSD test), $p \leq 0.05$, SE = standard error

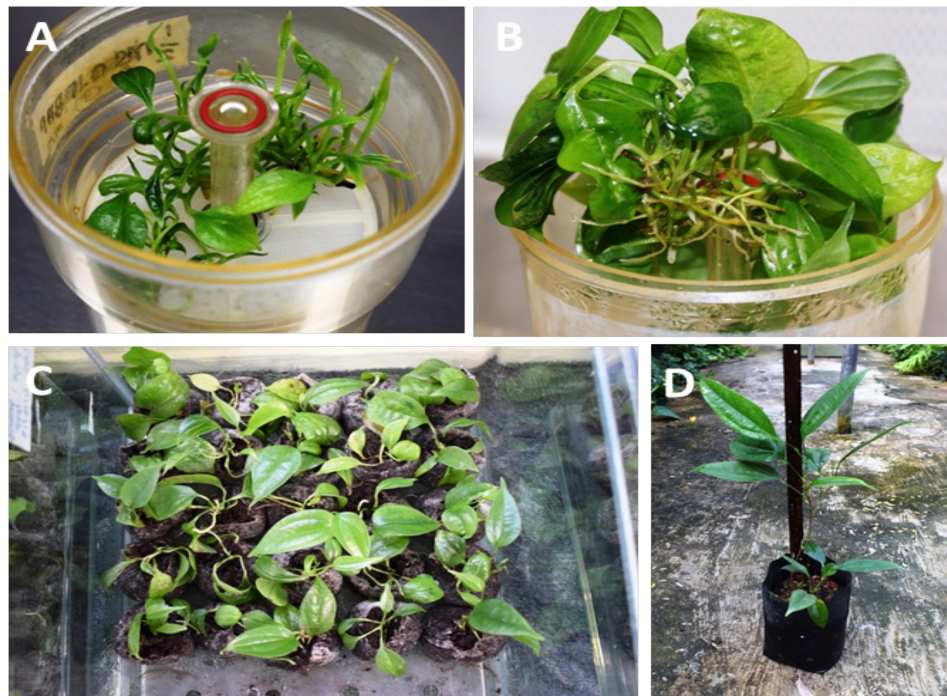


Figure 2 Tissue culture-derived *Smilax myosotiflora* plantlets using a temporary immersion system (TIS). The figures show the production of *Smilax myosotiflora* plantlets using a temporary immersion system (TIS) at different stages; (A) shoot multiplication, (B) root induction, (C) survived *Smilax myosotiflora* plantlets after acclimatisation, (D) *Smilax myosotiflora* tissue culture-derived plantlets in the nursery

inside the culture vessel and hyperhydricity. The use of the TIS reduces the tendency for hyperhydricity and high relative humidity because the atmosphere can be renewed despite the use of liquid media. Artificial ventilation is one of the measures taken to control hyperhydricity in several species of cultures (Polivanova & Bedarev 2022). The regulation of immersion frequency and exposure periods can also help reduce the problem of hyperhydricity that can lead to STN (Isah 2015).

Root induction

Table 4 shows that half-strength MS semi-solid medium with 0.5 mg/L IBA achieved the highest rooting (60%) in *S. myosotiflora* (Figure 1), with greater root number and length than all other treatments, including the control. In contrast, 3.0 mg/L NAA or IBA reduced rooting compared to the control. Although high auxin levels are often associated with callus formation that can inhibit root development, no callus was observed

Table 4 Root induction of *Smilax myosotiflora* shoots in half-strength semi-solid media supplemented with different NAA and IBA concentrations after 4 weeks in culture

PGR concentration (mg/L) in MS semi-solid media	Percentage of explant producing root (%)	Mean number of root ± SE	Mean root length (cm) ± SE
½ MS0	40	2.30 ± 0.39^{a}	0.86 ± 0.23^{a}
½ MS + 0.5 mg/L NAA	40	1.90 ± 0.41^{a}	0.76 ± 0.19^{a}
½ MS + 1.0 mg/L NAA	56	1.79 ± 0.19^{a}	0.64 ± 0.09^{a}
½ MS + 3.0 mg/L NAA	28	2.87 ± 0.67^{a}	0.78 ± 0.11^{a}
½ MS + 0.5 mg/L IBA	60	2.36 ± 0.40^{a}	1.05 ± 0.19^{a}
½ MS + 1.0 mg/L IBA	48	1.83 ± 0.21^{a}	0.72 ± 0.15^{a}
½ MS + 3.0 mg/L IBA	38	3.33 ± 1.05^{a}	0.69 ± 0.18^{a}

Note: Means with different letters in each column are significantly different (Tukey's HSD test), $p \leq 0.05$, SE = standard error

at any concentration. The reduced rooting at 3.0 mg/L is therefore more likely due to auxin-induced ethylene accumulation, toxicity, or hormonal imbalance, which can impair root initiation and elongation even without visible abnormalities (Bai et al. 2020).

The rooting behavior observed in *S. myosotiflora*, where lower concentrations of IBA (e.g., 0.5 mg/L) resulted in higher rooting percentages and longer roots compared to higher concentrations, is consistent with findings in *S. zeylanica*. In the latter, the optimal response was recorded at 2.0 μM IBA, which is approximately equivalent to 0.41 mg/L, and also produced high rooting efficiency without callus formation (Azad et al. 2022).

Since ½ MS with 0.5 mg/L IBA enhanced rooting in semi-solid culture, it was tested in liquid media (TIS) along with ¼, ¾, and full-strength MS (Table 5, Figure 2B). In TIS, rooting was lowest in ½ MS with 0.5 mg/L IBA, while ¼ MS with 0.5 mg/L IBA achieved the highest rooting (32%)—still below the semi-solid control. Root number per shoot (≤3) and root length (~1 cm) were similar across both culture types (Table 5). Lowering salt concentrations and adding auxins can promote root development in many plants, partly through stimulating root meristem activity under nutrient or water limitation (López et al. 2018). This pattern was also observed in *S. corbularia*, where ¼ MS with auxins supported faster root growth than higher-salt media (Jirakiattikul et al. 2013). For *S. myosotiflora*, however, the culture type—particularly semi-solid—had a greater influence on rooting than salt concentration.

Although root number (≤3) and length (~1 cm) were similar in both systems, rooting efficiency declined in TIS. This may relate to physiological and environmental factors, particularly immersion frequency, which affects the balance between nutrient uptake and aeration (Carvalho et al. 2019, Etienne & Berthouly 2002). Excessive immersion can create hypoxic conditions, limiting oxygen supply to root meristems (Edelmann 2022). Unlike semi-solid media, where explant bases contact moist, aerated gel, liquid cultures may submerge tissues too frequently, reducing gas exchange (Bai et al. 2020). In this study, 6 immersions per day for 5 minutes likely intensified these effects, lowering root induction compared to semi-solid culture.

Based on our observations, *S. myosotiflora* shoots were difficult to root in early culture experiments, taking over a month for roots to emerge despite the presence of rooting hormones in both semi-solid and liquid media. In the TIS vessel, where explants were positioned horizontally, the entire shoot was exposed to the rooting PGR-containing media, leading to root development along the nodes rather than at the shoot base, as seen in semi-solid media (Figure 2B). This posed challenges during the acclimatization stage, as the plantlets needed to be planted with their root system correctly positioned in the Jiffy 7®. The node-based root growth from TIS made acclimatisation difficult. Additionally, no rhizome development was observed in *S. myosotiflora* roots in either semi-solid or liquid media (TIS). While rhizome formation was absent under all culture conditions, the rhizome remains the primary medicinally valuable part of *S. myosotiflora*. *In vitro* rhizome induction could eventually support large-scale production and consistent raw material supply. However, given the species' climbing habit and strong nodal propagation response, nodal segments remain the most effective micropropagation method at present.

Table 5 Root induction of *Smilax myosotiflora* shoots in liquid media (TIS) in MS of different basal strengths supplemented with 0.5 mg/l IBA after 4 weeks in culture

PGR concentration (mg/L) in MS liquid media	Percentage of explant producing root (%)	Mean number of root ± SE	Mean root length (cm) ± SE
¼ MS + 0.5 mg/L IBA	32	2.88 ± 0.3[a]	1.02 ± 0.13[a]
½ MS + 0.5 mg/L IBA	12	2.33 ± 0.11[b]	0.88 ± 0.10[ab]
¾ MS + 0.5 mg/L IBA	13	1.40 ± 0.11[b]	0.20 ± 0.08[b]
MS + 0.5 mg/L IBA	13	1.00 ± 0.11b	0.20 ± 0.08[b]

Note: Means with different letters in each column are significantly different (Tukey's HSD test), $p \leq 0.05$, SE = standard error

Plantlet survival *ex vitro*

In the first acclimatisation trial, no plantlets survived due to fungal infection, which appeared after 2–3 weeks and reached 100% by week four in both semi-solid and TIS-derived plantlets. For the subsequent trial, plantlets and chamber walls were sprayed with 0.1% (w/v) Thiram 80WP at the start, and acclimatisation duration was shortened from 4 to 3 weeks. These changes increased survival, with TIS-derived plantlets achieving the highest rate (96%) despite being mostly non-rooted (Table 6). Figure 2C shows acclimatised plantlets in Jiffy 7® media under partial shade at ~90% relative humidity and 29 °C, conditions optimal for tropical species. The additional month of *in vitro* rooting can be omitted if TIS-derived plantlets are treated with rooting hormone *ex vitro*. Although these plantlets had fewer roots than those from semi-solid media, they showed higher survival. In this study, Seradix® No. 3 (~0.8% w/w IBA) was applied to the basal end before transplanting. Similar *ex vitro* rooting with IBA improved survival in *Passiflora foetida* (300 mg/L), *Artemisia absinthium* (200 mg/L), and *Aerva lanata* (200 mg/L) (Shekhawat et al. 2015, Shekhawat & Manokari 2015, Shekhawat et al. 2017). Combining TIS with *ex vitro* rooting can thus simplify *S. myosotiflora* micropropagation while reducing production time and cost.

The high survival rate of *S. myosotiflora* plantlets cultured in the temporary immersion system (TIS) is likely due to their enhanced adaptability to external conditions. In TIS, changes in airflow may promote the development of more functional stomata—small openings in plant tissue that facilitate gas exchange. It is known that the structure of the leaves of tissue-culture-produced plantlets differed from that of seedlings that were developed *ex vitro*, where the stomata were still not entirely functional to control the process of water vaporisation, especially plantlets that grow in semi-solid media. In addition to immature stomata function, plantlets grown from tissue culture have low levels of epicuticle wax formation and photosynthetic tissue formation in their leaves (Monja-Mio et al. 2015). When subjected to low humidity and high temperatures, immature stomata function will cause the plantlets to wither quickly, resulting in a low survival rate. The survivability of plantlets during acclimatisation can be increased by choosing a low nutrient concentration, which in this study was ½ MS and ¼ MS basal medium during the root induction process, resulting in low osmotic pressure and facilitating the plants' response during acclimatisation (Jirakiattikul et al. 2013).

Table 6 Survival percentage of *Smilax myosotiflora* from both media types after 3 weeks of acclimatisation

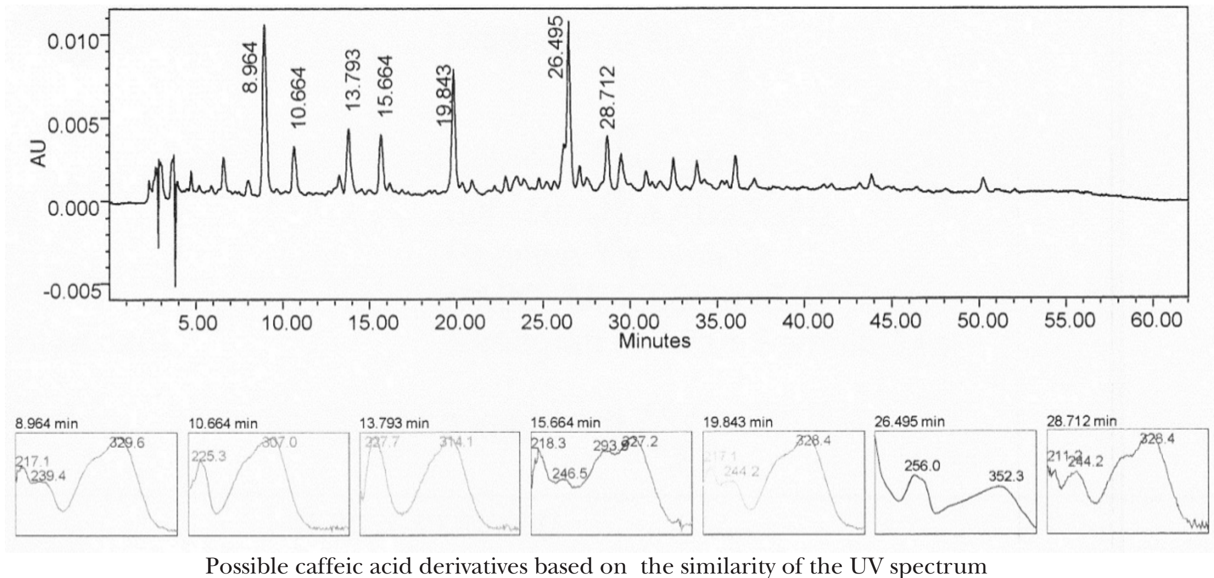
Source of Plantlets	Survival percentage after acclimatization (%)
Semi-solid media	52
Liquid media	96

Note: N = 50

The acclimatised plantlets were transferred into a soil mixture of baked soil and peat moss (2:1). During our observation, *S. myostiflora* preferred to grow under shade, with better growth and larger, healthier leaves. The trailing vines were directed to a stand or bench for the shoots to expand. Figure 2 (D) shows *S. myosotiflora* tissue culture-derived plantlets in our nursery.

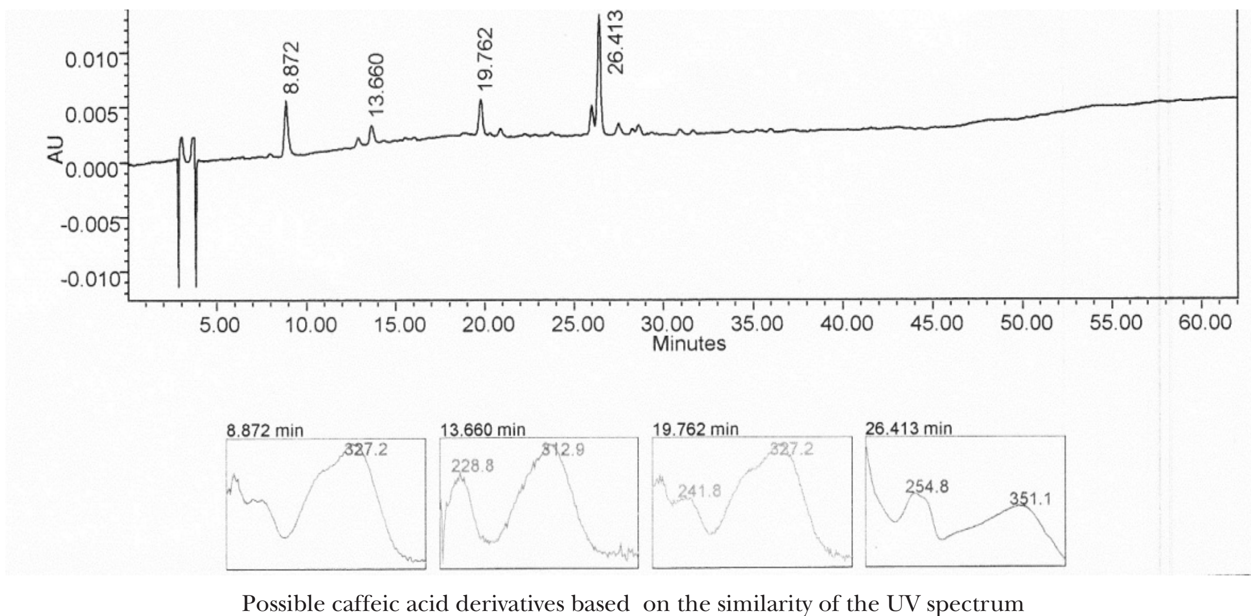
Primary biochemical profile of leaves from *in vitro* and *ex vitro* plantlets

The leaves of *in vitro S. myosotiflora* (from both semi-solid and liquid media, TIS at the shoot multiplication stage) and *ex vitro* plantlets (nearly 1-year-old after being transplanted into polybags) were harvested and tested for possible production of the bioactive compound. The first screenings of HPLC analysis on both samples have resulted in chromatogram profiles with a few peaks with a similar pattern on the UV spectrum, indicating possible bioactive compounds at retention times (RT) of 8.8, 15.6, 19.8 and 28.7 (*in vitro*) and RT 8.8 and 19.8 (*ex vitro*) (Figure 3, Figure 4). Further investigation has brought the result closer to a polyphenol, possibly a caffeic acid derivative compound that exhibits many health benefits associated with its antioxidant properties, including the prevention of inflammation, cancer, neurodegenerative diseases, and diabetes (Genaro-Mattos et al. 2015, Huang et al. 2013, Tosovic 2017). However, in the second screening using caffeic acid as the standard, no caffeic acid was found



Possible caffeic acid derivatives based on the similarity of the UV spectrum

Figure 3 HPLC profile of *Smilax myosotiflora* leaves from plantlets cultures *in vitro*



Possible caffeic acid derivatives based on the similarity of the UV spectrum

Figure 4 HPLC profile of *Smilax myosotiflora* leaves from plantlets grew in the nursery (*ex vitro*)

in either the *in vitro* or *ex vitro S. myosotiflora* sample extracts. Further investigation needs to be carried out to identify the caffeic acid derivatives. According to Fonseca et al. (2017), the polyphenol compound chlorogenic acid, the ester of caffeic acid with quinic acid, was found in an ethanol extract of *S. brasiliensis* leaves. In this study, the bioactive compounds in *in vitro* or *ex vitro S. myosotiflora* from tissue culture were probably present, but at a low concentration since the plantlets were grown in a controlled environment. Bioactive compounds, also referred to as secondary metabolites, are normally increased under induced conditions, such as during plant defence against biotic or abiotic stress.

CONCLUSIONS

Shoot multiplication and complete plant regeneration using both semi-solid and liquid media (TIS) demonstrated viable propagation methods for *S. myosotiflora*. The highest shoot multiplication rate (3.0 shoots/explant) was observed in the TIS, indicating its potential for large-scale propagation. During the rooting stage, semi-solid media supported greater root formation, while liquid culture (TIS) resulted in a higher survival rate during acclimatisation (96%), despite producing fewer roots. This suggests that the *in vitro* rooting stage can be omitted for TIS-derived plantlets, provided *ex vitro* rooting is applied. Further optimisation of the liquid system is recommended to encourage basal root development for easier transplantation. In addition, elicitation strategies using compounds such as methyl jasmonate, salicylic acid, and chitosan may be explored to enhance the production of bioactive compounds with antioxidant and phytoestrogenic activities *in vitro*. Overall, these findings contribute valuable insights into the tissue culture propagation of *S. myosotiflora* and offer a platform for future research into its phytochemical enhancement and commercial exploitation.

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