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EFFECTIVENESS OF META-TOPOLIN IN *IN VITRO* PROPAGATION AND GENETIC STABILITY ASSESSMENT OF MEDICINAL AND AROMATIC PLANTS

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Abstract

Meta-Topolin (mT), a compound from the cytokinin class that promotes plant growth and development, is characterized by lower toxicity than other cytokinins. This study explores the effect of mT on the in vitro multiplication of Hypericum perforatum L., Mentha x piperita L., and Stevia rebaudiana Bertoni. After six subcultures on Driver and Kuniyuki Walnut (DKW) medium supplemented with 2 mg/L mT, growth parameters were evaluated, including the number of shoots per explant, shoot length, the number of roots per explant, and root length. In the presence of mT, S. rebaudiana produced the highest number of shoots per explant (4.2 ± 0.12), while the longest shoots were recorded in M. piperita (6.2 ± 0.68 cm). The highest in vitro rooting percentage was observed in M. piperita, reaching 80%. The rooted shoots were acclimatized in perlite. Molecular analysis using Start Codon Targeted (SCoT) and Inter simple sequence repeat (ISSR) markers confirmed the genetic fidelity of the acclimatized plants compared to the mother plants, affirming the stability of in vitro cultures using mT as a cytokinin source.

Key words*: Hypericum perforatum, Mentha x piperita, ISSR, SCoT, Stevia rebaudiana.*

INTRODUCTION

Natural compounds derived from medicinal and aromatic plants (MAPs) have gained recognition as respected alternatives to synthetic drugs and as valuable resources for various industries, including cosmetics, food, feed, and environmental protection (Fierascu et al., 2021).
According to the World Health Organization, approximately 80% of the global population relies on traditional herbal medicines, driving a significant increase in the demand for medicinal plants worldwide (Radomir et al., 2023).
Hypericum perforatum L., commonly known as St. John's Wort, belongs to the *Hypericum* genus, which includes over 484 species (Kapoor et al., 2023). It is a herbaceous perennial plant native to Europe, Western Asia, and Northern Africa (Mohagheghzadeh et al., 2023). This species demonstrates various medicinal properties, such as antibacterial, antiviral, anti-inflammatory, and anticancer activities, attributed to its bioactive compounds, including hypericin, pseudohypericin, hyperforin, flavonoids, and phenolic compounds (Shasmita et al., 2023).
Mentha x piperita L., commonly referred to as peppermint, is a perennial herbaceous plant from the Lamiaceae family. It holds significant global importance as one of the leading essential oil crops. This species is highly valued for its rich content of secondary metabolites, which contribute to its distinctive aroma, flavor, and therapeutic properties. The secondary metabolites of peppermint are associated with various medicinal properties, including antioxidant, antimicrobial, anti-inflammatory, and anticancer activities (Hudz et al., 2023; Haddou et al., 2023; Afkar, 2024).
Stevia rebaudiana Bertoni, a perennial shrub belonging to the Asteraceae family, is renowned for its high content of steviol glycosides, which serve as a non-sucrose, calorie-free sweetener widely used in food products.

Beyond these sweet compounds, stevia leaves are rich in carbohydrates, lipids, dietary fibers, essential oils, water-soluble vitamins, minerals, and phenolic compounds.

Due to its diverse phytoconstituents, stevia exhibits a wide range of biological activities, including antidiabetic, antihypertensive, antimicrobial, anti-inflammatory, antitumor, and antioxidant properties (Chakma et al., 2023; Papaefthimiou et al., 2023; Śniegowska et al., 2024).

With the growing demand for herbal products, plant tissue culture emerges as a vital tool, offering an efficient alternative for the production of bioactive compounds and secondary metabolites. Consequently, it represents a promising strategy for conserving biodiversity while addressing the increasing global demand for medicinal plants and their derivatives (Akın, 2020).

Plant growth regulators (PGRs) and the multiple subcultures involved in the micropropagation process can lead to somaclonal variation. Therefore, the correct selection of PGRs and the assessment of genetic homogeneity are crucial aspects of micropropagation methods (Chirumamilla et al., 2021). Meta-Topolin [mT 6-(3-hydroxybenzylamino) purine], an aromatic cytokinin isolated from poplar leaves, stands out for its ability to promote shoot regeneration, delay senescence, and prevent necrosis, making it a valuable tool in micropropagation due to its beneficial effects on plant growth and proliferation *in vitro* (Gantait & Mitra, 2021).

The objectives of this study were to investigate the effects of mT on the *in vitro* growth and development of *H. perforatum*, *M. piperita*, and *S. rebaudiana*, as well as to assess the genetic variation that may have arisen among the clones of these three species using Start Codon Targeted (SCoT) and Inter simple sequence repeat (ISSR) molecular markers.

MATERIALS AND METHODS

Micropropagation

The source of plant material was potted plants of *H. perforatum*, *M. piperita*, and *S. rebaudiana*.

The young shoots for *in vitro* culture initiation were harvested from the mother plant and cut into fragments, each containing 2-3 nodes. The cuttings were thoroughly washed, first under running tap water and then with distilled water using a magnetic stirrer to remove impurities. Next, the shoot fragments were disinfected with a 20% bleach solution prepared from ACE (Procter and Gamble, Bucharest, Romania; <5% active ingredient) for 15 minutes, followed by triple rinsing with sterile distilled water. Single-node explants were inoculated onto Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) without plant growth regulators (PGRs) and solidified with 5 g/L (w/v) plant agar in glass test tubes (11.5 × 2 cm ø) containing 5 mL of sterile medium. During the initiation phase, 30 explants per species were inoculated (one explant per test tube). After four weeks, the percentage of successful *in vitro* initiation was calculated.The regenerated shoots were subcultured every four weeks for six passages on Driver and Kuniyuki Walnut (DKW) medium (Driver & Kuniyuki, 1984) supplemented with 2 mg/L mT and solidified with 5 g/L (w/v) plant agar. All components were procured from Duchefa Biochemie BV (Haarlem, The Netherlands). The pH of the media was adjusted to 5.8 before adding agar, and all culture media was autoclaved at 120 °C for 20 minutes. In this stage, 720 mL culture jars (13.5 × 9 cm ø) with screw polypropylene caps were used as culture vessels. Each jar contained 100 mL of sterile medium, and five stem explants with 2-3 nodes were inoculated

The *in vitro* cultures were maintained at 23 ± 3 °C, under a light intensity of 32.4 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (Philips CorePro LEDtube 1200 mm 16W865 CG, 1600 lm Cool Daylight) with a 16 h/8 h day/night photoperiod.After six subcultures, growth parameters were evaluated, including the number of shoots per explant, shoot length, roots per explant, and root length.

The rooted plants, obtained after six subcultures on DKW culture medium supplemented with 2 mg/L mT, were acclimatized in perlite using mini-greenhouses (Versay, T1, dimensions 39 × 25 × 7.5 cm, PVC). For each species, 30 *in vitro* rooted plantlets were acclimatized (ten plantlets per replicate), and the percentage of acclimatization was determined after four weeks.

Genetic fidelity assessment of *in vitro* raised plants using ISSR and SCoT markers

To assess the genetic fidelity of micropropagated plants of *S. rebaudiana*, *M. piperita,*

and *H. perforatum* to their mother plants, DNA was extracted from both the mother plant and one set for each molecular marker system used, which consisted of four *in vitro* plants that were randomly selected after four weeks of acclimatization in a mini-greenhouse. Biological material consisting of freshly harvested leaves was used for the genetic analyses.

DNA extraction

The extraction of total genomic DNA was performed using a DNeasy Plant Pro kit (Qiagen, Germany) following the protocol described by the supplier company.

PCR amplification

SCoT analysis

For the SCoT analysis, the PCR amplifications were carried out using the protocol described by Collard & Mackill (2009), with some modifications to the number of PCR amplification cycles and annealing temperature, depending on the primer selected (Hârţa et al., 2022; Samiee et al., 2023).

ISSR analysis

For the ISSR analysis, five ISSR primers were selected and PCR reactions were performed according to the protocol described by Morshedloo et al. 2014.

The SCoT and ISSR primers used in this study are shown in Table 1.

Table 1. The list of SCoT and ISSR primers selected for genetic stability analysis

Primer name	The 5'-3'nucleotide sequence of the Primer
SCoT 14	ACGACATGGCGACCACGC
SCoT 15	ACGACATGGCGACCGCGA
SCoT 24	CACCATGGCTACCACCAT
SCoT 26	ACCATGGCTACCACCGGG
ISSR 2	CACACACACACACACARC
ISSR 9	ACACACACACACACACYG
ISSR 12	AGAGAGAGAGAGAGAGT
ISSR 14	AGAGAGAGAGAGAGA GT
ISSR 32	CCCGTGTGTGTGTGTGT

The PCR-amplified products were separated by electrophoresis in agarose gels, under the conditions described in a previous study by Hârţa et al., 2024. To ensure the reproducibility of the results, PCR amplifications were repeated twice for each SCoT and ISSR primer.

Data analysis

One-way ANOVA followed by Tukey's HSD posthoc test ($P \leq 0.05$) was performed to determine the statistically significant differences between the mean values of the analysed morphological characteristics of vitro-plants. Values shown are means ± SE. For molecular markers analysis, gel images were evaluated using TL120 software (Nonlinear Dynamics, Newcastle upon Tyne, UK) to determine the molecular weight (bp) range of PCR amplified products. The number of amplified SCoT and ISSR monomorphic bands was also evaluated in this study.

RESULTS AND DISCUSSIONS

Micropropagation

In vitro culture initiation rates for *H. perforatum*, *M. piperita,* and *S. rebaudiana* on MS culture medium without PGRs were 64.44%, 68.89%, and 84.44%, respectively. The shoots obtained in the initiation stage were then maintained on the DKW culture medium supplemented with 2 mg/L mT for six subcultures of one month each (Figure 4). mT is an aromatic cytokinin known for its efficiency in shoot proliferation and root induction in economically important species. It is metabolized faster than conventional cytokinins and promotes plant growth and development, contributing to the alleviation of *in vitro* - induced morpho-anatomical and physiological disorders (Aremu et al., 2012; Ahmad & Anis, 2019; Souza et al., 2019; Elayaraja et al., 2019; Nowakowska & Pacholczak, 2020; Shekhawat et al., 2021; Manokari et al., 2021).

The presence of mT in the culture medium generated the highest number of shoots in *S. rebaudiana* (4.2 ± 0.12), while the longest shoots were observed in *M. piperita* (6.2 ± 0.46 cm) (Figures 1 and 2). It is noteworthy that, in all three species, the proliferated shoots did not exhibit morphophysiological abnormalities such as hyperhydric shoots, fragile leaves, or deformed structures. The beneficial effect of mT on the micropropagation of *S. rebaudiana* has also been reported when *in vitro* culture was conducted in RITA bioreactors (Ptak et al., 2023).

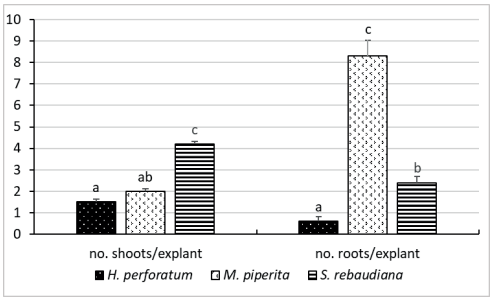

Figure 1. Number of shoots and number of roots per initial inoculum of *H. perforatum, M. piperita*, and *S. rebaudiana* after four weeks

In addition, DKW culture medium supplemented with 2 mg/L mT generated root formation in all three species. The highest rooting percentage was observed in *M. piperita* (80%), followed by *S. rebaudiana* (60%) and *H. perforatum* (40%). The highest number of roots per explant was 8.3 in *M. piperita*, followed by *S. rebaudiana* with 2.4 roots per explant (Figure 1). The lowest number of roots per explant was recorded in *H. perforatum* (0.6 ± 0.23), which also had the shortest roots, with an average length of 0.9 ± 0.32 cm.

Healthy explants, a good multiplication rate, and spontaneous rooting were also reported for *Aloe polyphylla* at a concentration of 5.0 mM mT (Bairu et al., 2007), at *Pelargonium* × *hortorum* (Mutui et al., 2012) and *Syzygium cumini* (Naaz et al., 2019).

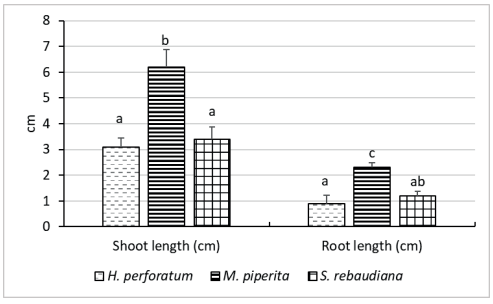

Figure 2. The length of shoots and roots of *H. perforatum, M. piperita*, and *S. rebaudiana*

The *in vitro* shoots rooted and then acclimatized *ex vitro* in perlite exhibited a 91.11% survival rate in *S. rebaudiana*, 90% in *M. piperita*, and 66.66% in *H. perforatum* (Figures 3 and 5). The present results are in accordance with those obtained in other studies conducted on *M. piperita* micropropagation (Sharma et al., 2019; Zayova et al., 2021), *S. rebaudiana* (Yücesan et al., 2016; Rodriguéz-Páez et al., 2024), and *H. perforatum* (Fascella et al., 2015).

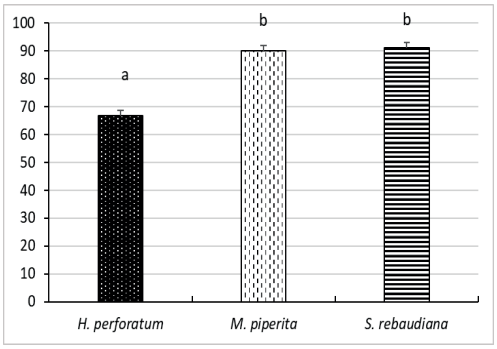

Figure 3. Perlite acclimation percentages

Genetic fidelity analysis

SCoT analysis. In total, 12 SCoT primers were used for the initial screening between the mother plants and the four randomly selected plants resulting from *in vitro* culture of each analysed medicinal and aromatic species. Four SCoT primers generated reproducible bands and were then used for genetic stability. The scorable bands for each SCoT primer varied from 7 (SCoT 24; SCoT 26) to 15 (SCoT 14). As can be seen in Table 2, the number of weight range of amplified bands (bp) varied between 300-2000 bp in *S. rebaudiana*, 300-2200 bp in *M. piperita*, and 300-2500 bp in *H. perforatum*. The highest total number of monomorphic SCoT bands was recorded in *H. perforatum*, namely 42, while the four selected SCoT primers generated 36 distinct and reproducible bands in *S. rebaudiana* (Table 2).

In this study, SCoT primers generated monomorphic PCR amplification products in all selected *in vitro* cultured plants, and no polymorphism was detected during SCoT analysis (Figure 6). Our results were consistent with those reported in previous studies (Tikendra et al., 2021; Biswas & Kumar, 2023; Bisht et al., 2024) which support that, when *in vitro* subcultured plants are used as commercial end products, assessing the genetic stability of acclimatized plants is essential.

ISSR analysis. According to Bisht et al. (2024) ISSR investigations use non-coding regions of DNA to assess genetic variability, diversity, and stability. Thus, the results of this study showed that ISSR markers are suitable for evaluating the

genetic fidelity of the analysed plant material grown under *in vitro* conditions.

A total of 11 ISSR primers were screened for genetic fidelity analysis of stevia, mint, and St. John's wort plants derived from *in vitro* shoot culture of which five ISSR primers produced reproducible monomorphic bands ranging from 300 to 3000 bp (Table 2). Maximum monomorphic bands (12) were produced by ISSR 12 primer in *H. perforatum*, whereas, minimum number of monomorphic bands (6) were produced by primer ISSR 32 in *M. piperita* (Table 2). The vitroplants and mother plants had a high level of genetic uniformity (100 %) and no genetic variation was identified (Figure 7). Previous studies have also documented the observed genetic homogeneity between the *in vitro* raised plants and the mother plants (Monalisa et al., 2024; Saritha et al., 2024; Awere et al., 2024).

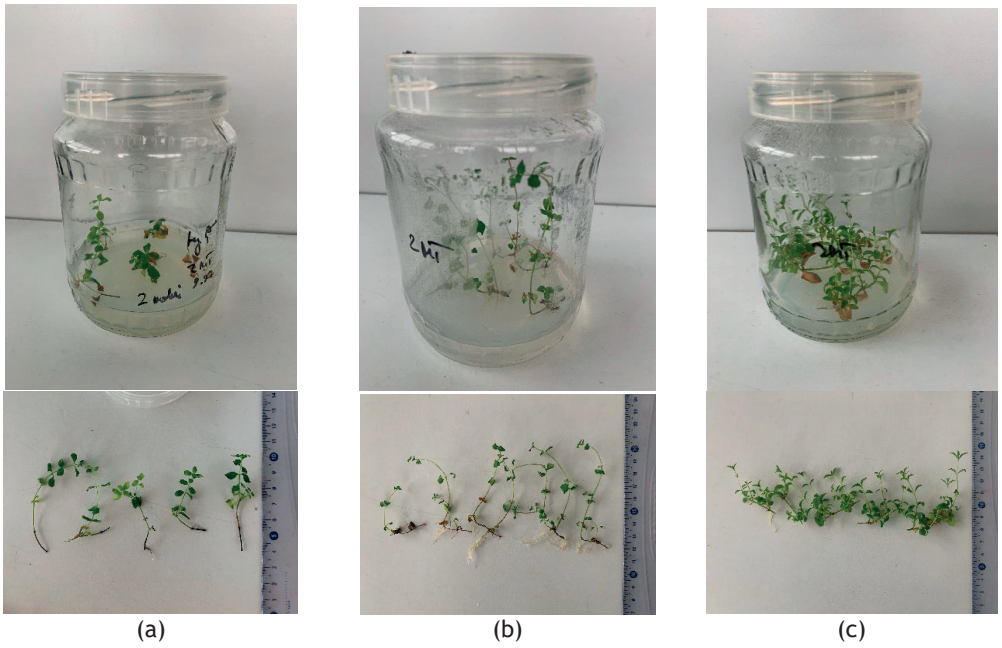

Figure 4. *In vitro* culture on DKW culture medium supplemented with 2 mg/L mT
(a) *H. perforatum;* (b) *M. piperita;* (c) *S. rebaudiana*

Figure 5. Acclimated plants: (a) *H. perforatum;* (b) *M. piperita;* (c) *S. rebaudiana*

Table 2. The number and size range (bp) of amplified SCoT and ISSR monomorphic bands

Primer name	Size range of monomorphic bands (bp)			Number of monomorphic bands		
	S. rebaudiana	*M. piperita*	*H. perforatum*	*S. rebaudiana*	*M. piperita*	*H. perforatum*
SCoT 14	400-2000	300-2200	450-1800	10	15	13
SCoT 15	300-1500	300-1700	300-1800	9	11	14
SCoT 24	350-1700	500-1500	500-1700	9	8	7
SCoT 26	400-2000	600-2000	550-2500	8	7	8
Total	-	-	-	36	41	42
ISSR 2	300-1500	400-2000	400-2000	7	8	10
ISSR 9	700-2200	500-1800	500-3000	8	10	11
ISSR 12	600-2000	800-3000	500-2200	10	8	12
ISSR 14	300-2500	400-1800	300-2200	7	8	10
Total	-	-	-	41	40	50

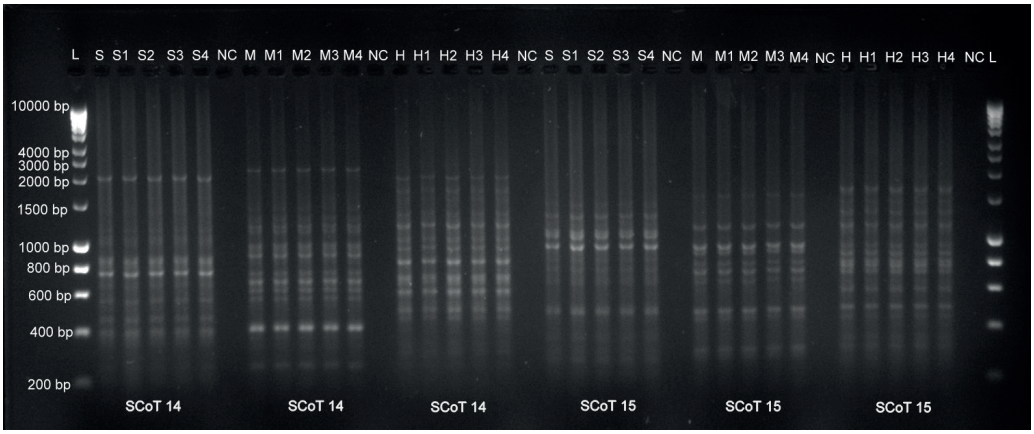

Figure 6. The SCoT profiles of mother plants and selected acclimated vitroplants of S. *rebaudiana* (S1-S4), *M. piperita* (M1-M4), and *H. perforatum* (H1-H4) generated by primers SCoT 14 and SCoT 15. Lanes: S, M, H – SCoT bands from the mother plants; L – molecular marker (1 Kb HyperLadder, Bioline, UK); NC – sample controls without DNA.

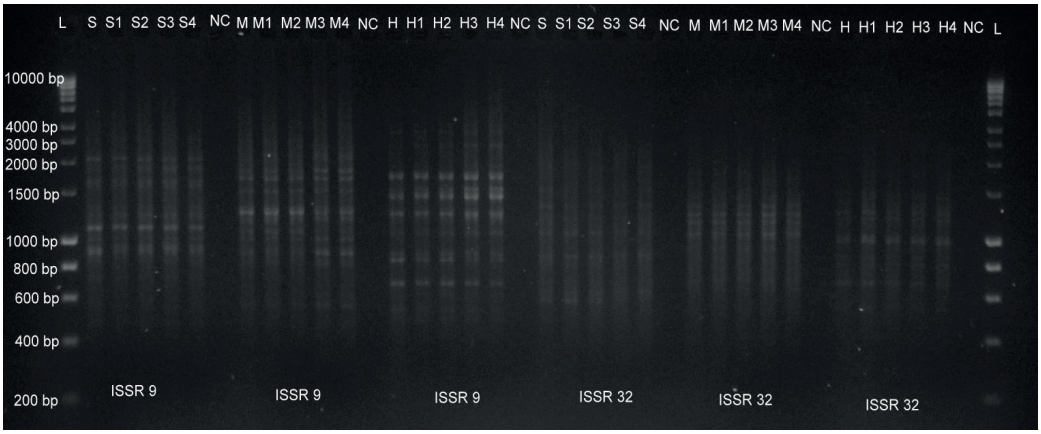

Figure 7. The ISSR profiles of mother plants and selected acclimated vitroplants of *S. rebaudiana* (S1-S4), *M. piperita* (M1-M4), and *H. perforatum* (H1-H4) generated by primers ISSR 9 and ISSR 32. Lanes: S, M, H – ISSR bands from the mother plants; L – molecular marker (1 Kb HyperLadder, Bioline, UK); NC – sample controls without DNA.

CONCLUSIONS

Applying mT during the *in vitro* multiplication stage of *H. perforatum*, *M. piperita*, and *S. rebaudiana* resulted in shoots free of morpho-physiological alterations and promoted root development, particularly in mint. Furthermore, genetic stability analysis using SCoT and ISSR markers revealed that the *in vitro*-raised plants were genetically true-to-type with the mother plants, confirming the stability of *in vitro* cultures when using mT as a cytokinin source.

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