Journal of Agricultural Sciences
(Tarim Bilimleri Dergisi)

J Agr Sci-Tarim Bili
e-ISSN: 2148-9297
jas.ankara.edu.tr

JAS

Cell Suspension Cultures and High Frequency Shoot Regeneration of Some Hypericum species

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ARTICLE INFO

Research Article
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Received: 19 August 2024 / Revised: 20 September 2024 / Accepted: 29 October 2024 / Online: 25 March 2025

Cite this article

Ahmed H A A, Uranbey S, Salaj T, Mistrikova V (2025). Cell Suspension Cultures and High Frequency Shoot Regeneration of Some Hypericum species. *Journal of Agricultural Sciences (Tarim Bilimleri Dergisi),* 31(2):319-331. DOI: 10.15832/ankutbd.1535826

ABSTRACT

The *Hypericum* genus is significant both medically and economically due to its bioactive compounds. This study utilized plant biotechnology techniques to develop an efficient and reliable adventitious shoot regeneration and suitable cell suspension culture system for various *Hypericum* species, including *Hypericum perforatum* L., *Hypericum leptophyllum* Hochst, *Hypericum heterophyllum* L., *Hypericum humifusum* L., and *Hypericum athoum* Boiss. & Orph. High frequency of callus induction using leaf explants on MS medium containing 1.0 mg/L BAP and 0.1 mg/L 2,4-D for *Hypericum perforatum* L. (100%), *Hypericum humifusum* L. (90%), and *Hypericum leptophyllum* Hochst (90%). The best shoot regeneration was also achieved in *Hypericum humifusum* L. (86.3%), *Hypericum perforatum* L. (73.0%), *Hypericum leptophyllum* Hochst (45.67%) and *Hypericum athoum* Boiss. & Orph. (18.33%) on MS medium with 1.0 mg/L BAP and 0.1 mg/L 2,4-D. Calli clusters obtained on callus induction medium were cultured on cell suspension culture MS basal media with B5 vitamins containing 2 mg/L glycine, 1 mg/L 2,4-D, 0.1 mg/L KIN, and 0.1 mg/L BAP. The friable calli developed in the suspension culture and plated on the medium. The proembryogenic structures formed turned to embryonic structures. These globular embryos further transformed into heart and cotyledonary stage and germinated. The maximum number of shoot/callus or percentage of germination of somatic embryo were respectively recorded for H. *perforatum* L. (15.37/callus), H. *leptophyllum* Hochst (6.9/callus), H. *heterophyllum* L. (9.6/callus), H. *humifusum* L. (16.8/callus) and H. athoum Boiss. & Orph. (11.7/callus). H. perforatum L. and H. humifusum L. showed the best shoot regeneration capacity as recorded in adventitious shoot regeneration studies. The plants were acclimatized with 85-100 % survival rat, H. perforatum and H. humifusum L. had also the highest survival rate (100%) as having regeneration capacity.

Keywords: Cell suspension culture, Shoot regeneration, *Hypericum* species, Medicinal plant

1. Introduction

Hypericum species belong to the Clusiaceae (Guttiferae, Hypericaceae) family and are distributed worldwide with around 500 species (Crockett & Robson 2011; Henzelyová & Čellárová 2018). *Hypericum* genus, used in the treatment of numerous diseases, is an important taxonomic group for its invaluable source of natural compounds with therapeutic properties (Alahmad et al. 2021; Shasmita et al. 2023). The some of these natural compounds has rich bioactive properties. Therefore, *Hypericum* species are also characterized by the presence of different types of secretory tissues such as dark glands and secretory ducts in their structures. These secretory structures are areas where bioactive substances are accumulated or synthesized and can be located in different places depending on the plant tissue. The most important of metabolites are hypericin and pseudohypericin known as compounds of the naphthodiantrone group, hyperforin and adhyperforin, known as phloroglucinol derivatives, flavonoids, xanthones, and procyanidin compounds (Barnes et al. 2001; Greeson et al. 2001; Dall'Agnol et al. 2003; Radusiene et al. 2004; Hong et al. 2004; Tanaka & Takaishi 2006; Smelcerovic et al. 2006; Medina et al. 2006; Uzbay 2008; Zubrická et al. 2015; Jendželovská et al. 2016). These compounds can only be synthesized by plants of the *Hypericum* genus and have important biological activities, including antiviral, anticancer, antiretroviral, antibacterial and antidepressant properties (Ciccarelli et al. 2001; Guedes & Eriksson 2005; Griffith et al. 2010; Crockett & Robson 2011). Due to the high medicinal and economic value of *Hypericum* species, the demand for biotechnological methods has increased. Many of the endemic *Hypericum* species are to be preserved and the production of secondary metabolites is possible in a short time and in large quantities using biotechnological methods (Simic et al. 2014; Hussain et al. 2022). Plant tissue, organ and cell culture applications focus on the presence of bioactive compounds and these techniques are employed under controlled environmental conditions to generate plant-specific metabolites used in medicinal and cosmetics industry (Eibl et al. 2018; Al-Atrakchii et al. 2019; Gubser et al. 2021; Faizy at al. 2022; Murthy et al. 2023; Abdulkareem et al. 2024). Cell suspension methods maybe

important tools for the extraction of secondary metabolites from medicinal plants. These techniques have been also becoming increasingly popular as a host system to produce recombinant proteins (Tekoah et al. 2015; Yue et al. 2016). Cell suspension methods can be used to produce virus-free, low-cost in plant and stable secondary metabolites production. In addition, they are effective on post-translational modifications and cost efficiency over bacterial expression systems (Santos et al. 2016; Zagorskaya & Deineko 2017; Permyakova et al. 2019). There are many factors influencing cell suspension culture and the size of cell aggregates such as plant species, explant source, type of explant, phytochemicals, dark/light conditions, temperature, nutrient composition, pH of the culture medium and sucrose concentration (Buter et al. 1998; Bais et al. 2002; Parveen & Shahzad 2014; Saad et al. 2016).

Tissue culture applications are an alternative method for the micropropagation of *Hypericum* species such as H. *perforatum* (Santarem & Astarita 2003; Karpinen et al. 2006; Wójcik & Podstolki 2007; Palmer & Keller 2011; Banerjee et al. 2012; Afsharzaleh et al. 2021; Mikhovich et al. 2021; Ravindran et al. 2022), H. *heterophyllum* (Ayan & Cirak 2006), H. *humifusum* L. (Selvakesavan & Gregory 2021), H. *foliosum* Aiton, H. *hirsutum* L. and H. *maculatum* Crantz (Moura 1998; Coste et al. 2011). H. *perforatum*, H. *angustifolium* and H. *triquetrifolium* have been studied under *in vitro* conditions and they have been produced in large quantities (Pretto & Santarem, 2000; Mulinacci et al. 2008; Karakaş et al. 2009). Moreover, there are only a few reports on cell suspension cultures on *Hypericum* species (Selvakesavan & Gregory 2021; Kruszka et al. 2022). This study also aimed to improve callus induction and regeneration suitable for *in vitro* secondary metabolite production using cell suspension culture method in different *Hypericum* species (H. *perforatum* L, H. *leptophyllum* Hochst, H. *heterophyllum* L, H. *humifusum* L. and H. *athoum* Boiss. & Orph.) and to develop high frequency adventitious shoot regeneration system by organogenesis or somatic embryogenesis.

2. Material and Methods

2.1. Hypericum species

The seeds of H. *perforatum* L, H. *leptophyllum* Hochst, H. *heterophyllum* L. were collected from various agro-ecological regions in Türkiye. H. *humifusum* L. and H. *athoum* Boiss. & Orph. were also obtained from the laboratory of the Pavol Jozef Šafárik University, Faculty of Science, Institute of Biology and Ecology, Department of Genetics, Košice-Slovak Republic.

2.2. Surface sterilization and germination condition

The seeds of *Hypericum* species were kept at +4 ºC for 24 hours, and surface sterilization was carried out by soaking the seeds in 1% $AgNO_3$ for 15 min. The seeds were then washed five times in sterilized water for 5 minutes. Thereafter, the seeds were cultured on MS (Murashige & Skoog 1962) with Gamborg's B5 vitamins (Gamborg et al. 1968) containing 30 g/L sucrose, 7 g/L agar and 2 mg/L glycine in a growth cabinet at 24°C with 8/16 h dark/ fluorescent light.

2.3. Culture conditions and callus initiation from leaf explants of Hypericum species

The seeds of different *Hypericum species* were germinated, 7-10 old days of with length of 6-8.5 cm and then isolated and used to initiate callus and regenerate shoots. Various combinations and concentrations of 6-benzylaminopurine (BAP), Kinetin (KIN) and dichlorophenoxyacetic acid (2,4-D) were utilized to start callus induction and shoot regeneration. Culture on MS/Gamborg B5 vitamins medium with stable concentrations of growth regulators was conducted using *in vitro* seedlings of *Hypericum.* The effects of 2,4-D (0.1 mg/L and 1.0 mg/L) in combination with BAP (0.1 mg/L and 1.0 mg/L) and Kinetin (0.1 mg/L and 1.0 mg/L) were tested. The culture medium was supplemented with 30 g/L sucrose and solidified with 7 g/L agar. The pH of the medium was adjusted to 5.6 before autoclaving. All explants were cultured in the dark at a temperature of 24 ± 2 °C. The embryonic white, healthy, friable, lightly dispersed and granular calli were weighed in a sterile cabinet and on sterile blotting paper.

2.4. Initiation of cell suspension cultures

After germination of *Hypericum* species, 7-10 old days of leaf were used to initiate callus on MS medium supplemented with 1 mg/L 2,4-D, 0.1 mg/L BAP and 7 g/L agar. They were then transferred to 250 mL Erlenmeyer flasks containing 100 mL liquid medium. This medium consisted of MS basal media, Gamborg's B5 vitamins, 30 g/L sucrose, and 2 mg/L glycine. The medium was supplemented with 1 mg/L 2,4-D, 0.1 mg/L KIN, and 0.1 mg/L BAP.

The cell suspensions were then shaken at 120 rpm on an orbital shaker and cultured at 24 ± 1 °C under 40% relative humidity and continuous darkness, as this culture conditions promotes biomass production. The growth of the suspension cultures was assessed using sedimented cell volume (SCV) as a non-destructive quantitative measure. Every two weeks, subcultures were performed by transferring 3 mL of SCV from each sample into 22 mL of fresh liquid medium. This process was repeated three times with five Erlenmeyer flasks in each repetition. Erlenmeyer flasks were tightly sealed with sterile aluminium foil and wrapped with parafilm on the outermost part. To ensure proper dispersion and separation of the cells within

the suspension medium, the cell suspension culture media were kept in a growth chamber. Developed massive cell aggregates were transferred to the same media solidified with agar.

2.5. Light microscopy analysis

Microscopic analysis techniques were employed for shape assessment, facilitating the evaluation of cultured cell quality (the quality of cultured cells). Suspension cultures were observed with a Leica DM5500B microscope, equipped with a LEICA DFC450 C camera (Pragolab s.r.o., Slovak Republic); and calluses were observed with a LEICA MZ10F stereo microscope equipped with a LEICA DFC 420C camera (Pragolab s.r.o., Slovak Republic). This parameter is of particular importance, especially in scenarios involving size-able cell aggregates such as those found in somatic embryo cultures. Shape analysis facilitates the identification and elimination of aberrant somatic embryos during developmental stages, allowing the selection of somatic embryos demonstrating fitting attributes. Furthermore, investigations were conducted on samples derived from cell suspension cultures of Hypericum. The progression and growth of somatic embryos were examined.

2.6. Maturation of the somatic embryos and plant regeneration

Globular somatic embryos were cultured using different media formulations, including half strength basal medium solidified with agar without PGRs or with various doses of BAP (0.1 mg/L and 1.0 mg/L) and 2,4-D (0.1 mg/L and 1.0 mg/L). The cultures were kept in the dark at a temperature of 25± ◦C for 9 weeks, with regular transfers to fresh medium every 2 weeks. Each Petri dish (10 cm diameter) contained 4 globular somatic embryos as one replication. Once callus formation occurred, the embryos were conveyed to maturation medium MS + BAP 0.1 mg/L + sucrose 30 g/L and agar 7 g/L for shoot regeneration. Subcultures were performed every two weeks.

2.7. Rooting and acclimatization

Shoots developed adventitiously on calli were carefully excised using forceps and scalpels, and then carried to a MS medium containing 0.2 mg/L IBA for rooting. The cultures were placed under white fluorescent light with a photoperiod of 16 hours of light and 8 hours of darkness, at a controlled temperature of 25±2 °C. Once the plantlets had developed roots, any agar residues were gently washed off without causing damage to the roots. The rooted plantlets were then transplanted into a substrate consisting of peat and perlite mixed with field soil. To prevent moisture loss, all transplanted plants were covered with polyethylene bags. After 10 days, the bags were gradually opened to gradually expose the plants to the greenhouse environment and acclimatize them.

2.8. Experimental design

Regeneration trials were designed according to the completely randomised design with three replicates, using 90 mm disposable Petri dishes in which 10 explants were cultured in each replicate. The data obtained were analysed using the JMP-13 statistical package (SAS 2017). The mean values of the applications were compared using the Duncan test. Percentages were subjected to arcsine transformation before statistical analysis (Snedecor & Cochran 1967).

3. Results and Discussion

The seeds of *Hypericum* species were germinated on MS medium with a success rate ranging from 40% to 100%, and no contamination was observed. (Figure 1). H. *perforatum* L. and H. *athoum* Boiss. & Orph. showed high germination capacity. This germination variation of the species could be due to the provision of seeds from natural environments or different culture conditions. Germinated seeds were subcultured from auxiliary buds after 4-5 weeks. Developed plantlets produced mass branches and formed thick roots as shown (Figure 2). The leaf explants were isolated from these new plantlets in all species.

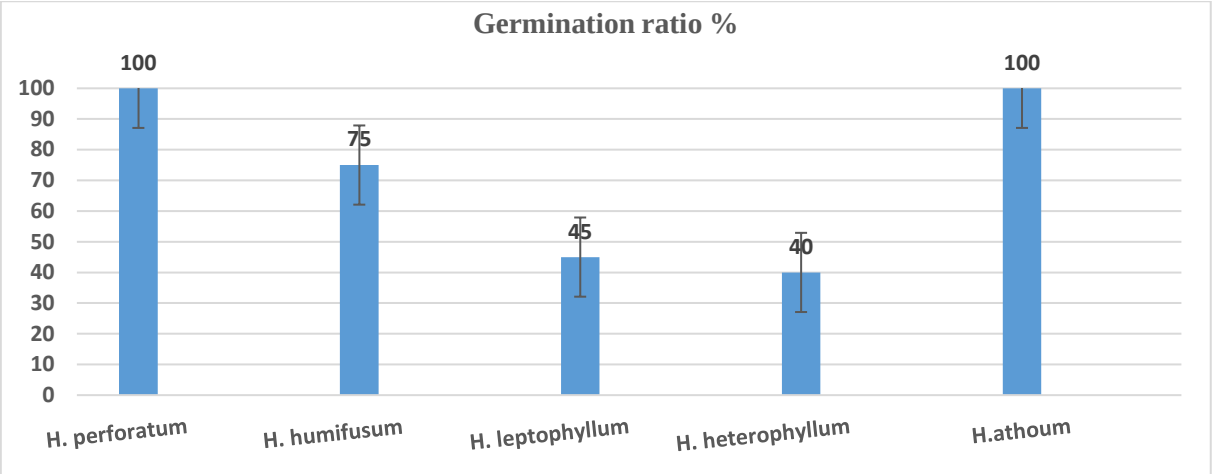

Figure 1- *In vitro* germination ratio of *Hyericum* species

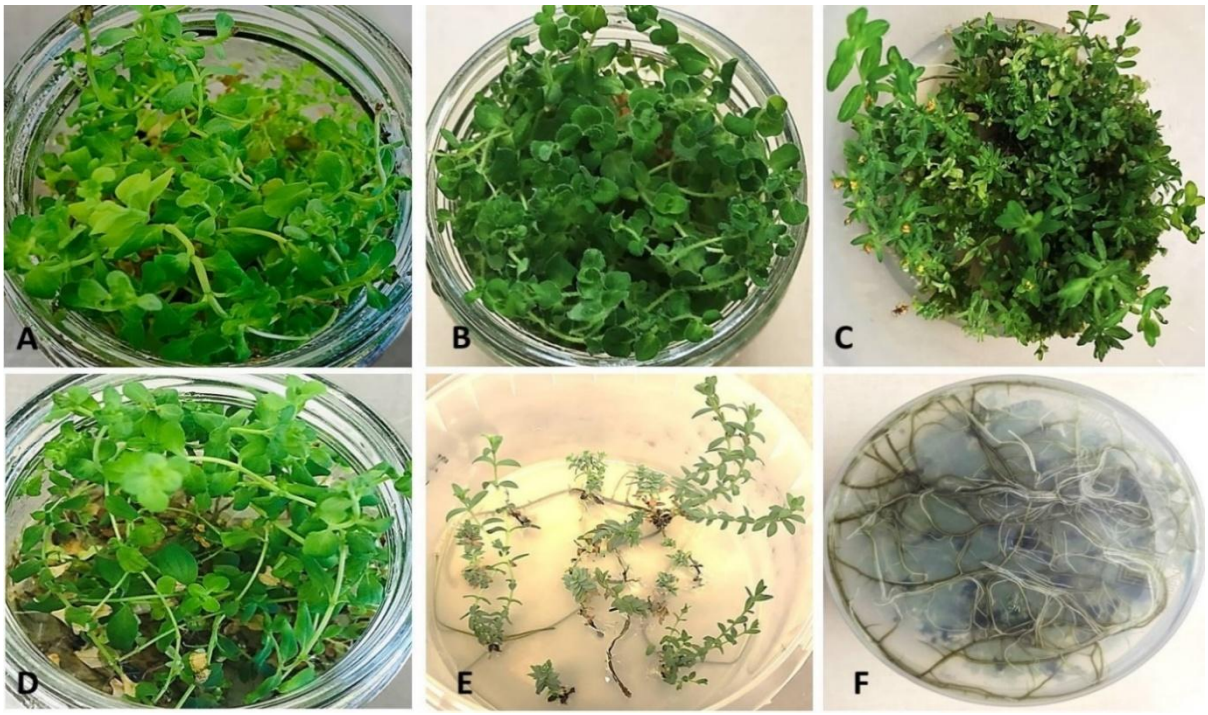

Figure 2- The leaf explants derived from the plantlets developing from auxiliary buds of germinated seeds after 4-5 weeks culture initiation (A*)* H. *perforatum* L. (B) H. *athoum* Boiss. & Orph. (C) H. *humifusum* L. (D) H. *leptophyllum* Hochst. (E) H. *heterophyllum* L. (F)Formation of thick and mass roots of germinated seeds of H. *perforatum* L

3.1. Adventitious shoot regeneration of Hypericum ssp

In the study, the effects of 2,4-D and two cytokinins (BAP and KIN) on the organogenesis of five *Hypericum* species cells were investigated. The leaf explants of all species were enlarged within 10 days. Callus formation and the induction of loose cell masses in all *Hypericum* species derived from leaf explants were also observed after two-four weeks of culture initation in all species under dark conditions. Clusters of calli started to emerge from the incisions of leaf and afterwards calli consistently developed from the cut zone of the petiole of the whole species. The embryonic white, healthy, friable, lightly dispersed and granular calli developed on leaf explants of the whole species (Figure 3). 2,4-D concentration of 0.1-1.0 mg/L with BAP or KIN on the media promoted the callus initiation responses of the whole species. The doses of 2,4-D drastically affected callus regeneration in many plant species (Zheng & Konzak 1999; Niazian et al. 2019). The best callus induction was seen on MS medium containing 1.0 mg/L BAP and 0.1 mg/L 2,4-D for H. *perforatum* L. (100%), H. *humifusum L.* (90.0%) and H. *leptophyllum* Hochst (90.0%). The highest callus weight was also determined on the same medium for three species Tables 1-3. However, 1.0 mg/L KIN and 0.1-1.0 mg/L 2,4-D promoted callus formation and callus weight for H. *athoum* Boiss. & Orph. and H. *heterophyllum* L. Tables 4-5. Similarly, combinations of KIN X 2,4-D were reported for suitable callus induction and MS medium including 0.90 µM of 2,4-D and 0.11 µM of KN showed the best callus induction from leaf explants of H. *perforatum* L. (Bais et al. 2002). Leaf, leaf discs and leaf axil derived from *in vitro* conditions for is generally the best source

for efficient and high frequency callus formation and shoot regeneration for *Hypericum* species (Pretto & Santarem 2000; Xu et al. 2001; Mccoy & Camper 2002; Pasqua et al. 2003; Ayan et al. 2005; Wójcik & Podstolki 2007; Mulinacci et al. 2008; Bais et al. 2002; Karakaş et al. 2015; Afsharzaleh et al. 2021; Ravindran et al. 2022). But limited studies were reported different organs and tissues such as mature leaf disc, seeds, mature stem segments, anthers of mature flower and *in vitro* nodal segments could be utilized for organogenesis via callus culture (Kirakosyan et al. 2000; Ayan et al. 2005; Mañero et al. 2012; Savio et al. 2012). In our study leaf explants also derived from *in vitro* responded well callus formation and shoot regeneration.

Then 4 weeks later globular calli with somatic embryos were transferred to the first same medium supplemented with 2,4-D BAP and KIN for shoot regeneration. Shoot primordia's initials occurred after 4-8 weeks of culture for all species tested. Number of shoots was recorded after 8 weeks of culture for each basic media and all species tested. The frequency of shoot regeneration was statistically influenced (P<0.01) for each basal media in all *Hypericum* species Table 1-5.

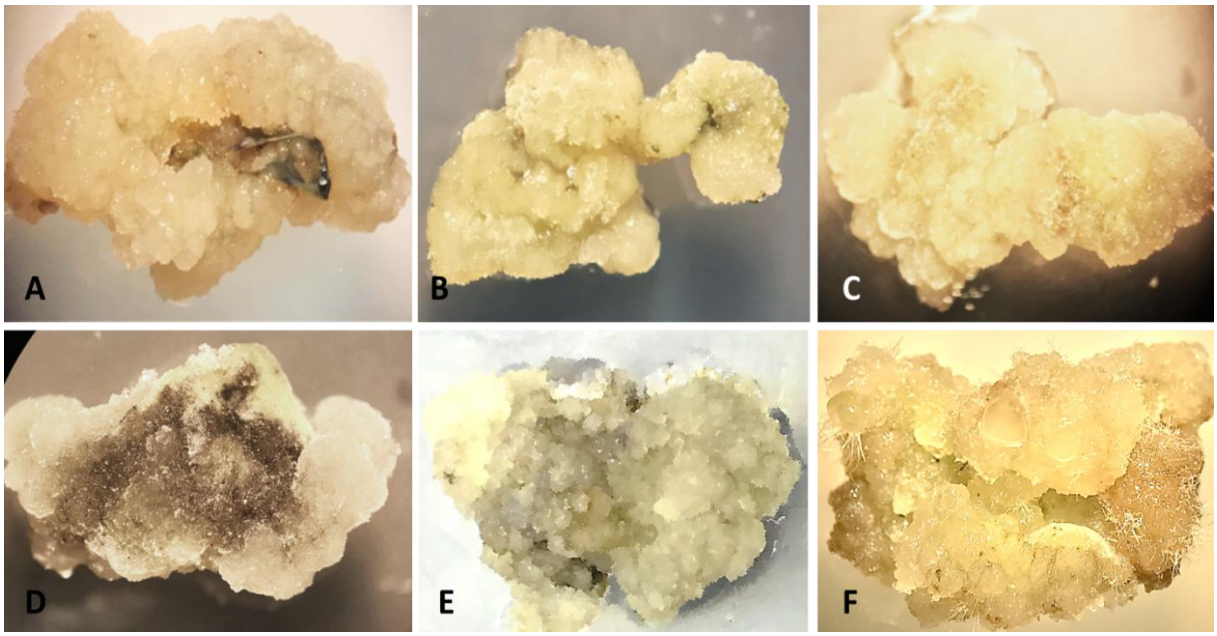

Figure 3- Callus formation and the induction of cell masses in all *Hypericum* species derived from leaf explants under dark conditions (A-B) H. *perforatum* L., (C) H. *athoum* Boiss. & Orph., (D) H. *humifusum* L. (E) H. *leptophyllum* Hochst, (F) H. *heterophyllum* L

Table 1- Effect of PGRs on callus and shoot induction from leaf explants of *Hypericum perforatum* L

PGR (mg/L)			*Leaf explants*			
BAP	***KIN***	***2,4-D***	***Callus induction (%)***	***Callus weight (g)***	***% Shoot Induction***	***Number of shoots/explants***
0.1		1.0	90.0 a*	2.103 ab*	57.33 b*	5.73 b*
1.0		0.1	90.0 a	2.750 ab	73.00 a	7.30 a
1.0		1.0	83.9 ab	3.043 ab	58.33 b	6.03 b
	0.1	1.0	70.8 abc	3.593 a	54.33 b	6.20 b
	1.0	0.1	59.7 c	1.427 b	23.67 c	3.31 c
	1.0	1.0	68.1 bc	1.613 b	32.67 c	4.02 c

BAP 6-benzylaminopurine, KIN: Kinetin, 2,4-D: dichlorophenoxyacetic acid; * Different letters in a column are significant at P<0.01 level

Table 2- Effect of PGRs on callus and shoot induction from leaf explants of *Hypericum humifusum* L

PGR (mg/L)			*Leaf explants*			
BAP	***KIN***	***2,4-D***	***Callus induction (%)***	***Callus weight (g)***	***% Shoot Induction***	***Number of shoots/explants***
0.1		1.0	75.00	0.873 b*	34.67 b*	4.57 c*
1.0		0.1	90.00	3.070 a	86.33 a	8.63 a
1.0		1.0	77.73	1.107 b	43.67 b	5.48 b
	0.1	1.0	63.87	0.038 c	17.33 c	2.73 d
	1.0	0.1	63.93	0.051 c	16.67 c	2.66 d
	1.0	1.0	71.60	0.036 c	13.67 c	2.57 d

BAP 6-benzylaminopurine, KIN: Kinetin, 2,4-D: dichlorophenoxyacetic acid; * Different letters in a column are significant at P<0.01 level

Table 3- Effect of PGRs on callus and shoot induction from leaf explants of *Hypericum leptophyllum* Hochst

PGR (mg/L)			*Leaf explants*			
BAP	***KIN***	***2,4-D***	***Callus induction (%)***	***Callus weight (g)***	***% Shoot Induction***	***Number of shoots/explants***
0.1		1.0	90.00 a*	2.83 c*	50.00 a*	5.00 ab*
1.0		0.1	90.00 a	5.95 a	45.67 ab	5.30 a
1.0		1.0	72.27 b	4.01 b	41.67 b	4.17 bc
	0.1	1.0	90.00 a	1.05 d	17.67 d	2.97 d
	1.0	0.1	81.13 ab	2.87 c	19.67 d	3.46 cd
	1.0	1.0	90.00 a	2.66 c	27.00 c	4.05 c

BAP 6-benzylaminopurine, KIN: Kinetin, 2,4-D: dichlorophenoxyacetic acid; * Different letters in a column are significant at P<0.01 level

Table 4- Effect of PGRs on callus and shoot induction from leaf explants of *Hypericum heterophyllum* L

PGR (mg/L)			*Leaf explants*			
BAP	***KIN***	***2,4-D***	***Callus induction (%)***	***Callus weight (g)***	***% Shoot Induction***	***Number of shoots/explants***
0.1		1.0	90.00 a*	0.037 c*	8.67 c*	2.17 b*
1.0		0.1	59.00 b	0.104 b	13.00 b	2.58 b
1.0		1.0	60.00 b	0.036 c	12.00 bc	2.57 b
	0.1	1.0	53.07 b	0.036 c	12.00 bc	2.57 b
	1.0	0.1	76.93 ab	0.081 b	18.33 a	3.25 a
	1.0	1.0	77.73 ab	0.293 a	14.00 b	2.80 ab

BAP 6-benzylaminopurine, KIN: Kinetin, 2,4-D: dichlorophenoxyacetic acid; * Different letters in a column are significant at P<0.01 level

Table 5- Effect of PGRs on callus and shoot induction from leaf explants of *Hypericum athoum* Boiss. & Orph

PGR (mg/L)			*Leaf explants*			
BAP	***KIN***	***2,4-D***	***Callus induction (%)***	***Callus weight (g)***	***% Shoot Induction***	***Number of shoots/explants***
0.1		1.0	61.93 b*	0.650 b*	15.00 b*	3.22 abc*
1.0		0.1	65.87 b	0.287 b	16.67 ab	3.82 a
1.0		1.0	61.93 b	0.553 b	7.67 c	2.61 d
	0.1	1.0	90.00 a	0.790 b	14.67 b	2.99 cd
	1.0	0.1	78.93 ab	0.847 b	14.67 b	3.33 abc
	1.0	1.0	90.00 a	3.523 a	23.00 a	3.65 ab

BAP 6-benzylaminopurine, KIN: Kinetin, 2,4-D: dichlorophenoxyacetic acid; * Different letters in a column are significant at P<P<0.01 level

All species formed high frequency shoot proliferation all media tested. H. *perforatum* L., H. *humifusum* L. and H. *leptophyllum* Hochst, showed high regeneration capacity in all media tested compared to H. *heterophyllum* L. and H. *athoum* Boiss. & Orph. The maximum shoot regeneration frequency (86.3 %, 73.0 %, 45.67 %, 18.33 % and 16.67 %) was respectively recorded in H. *humifusum* L., H. *perforatum* L., H. *leptophyllum* Hochst, and H. *athoum* Boiss. & Orph. on MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L 2,4-D. The highest number of shoots per explant (8.33, 7.30, 5.30, 3.82) was also respectively found on the same medium in H. *humifusum* L., H. *perforatum* L., H. *leptophyllum* Hochst, and H. *athoum* Boiss. & Orph. (Figure 4). However, the best regeneration frequency (18.33 %) and the maximum of shoots per explants (3.25) were determined on MS basal medium containing 1.0 mg/L Kinetin and 0.1 mg/L 2,4-D for H. *heterophyllum* Hochst.

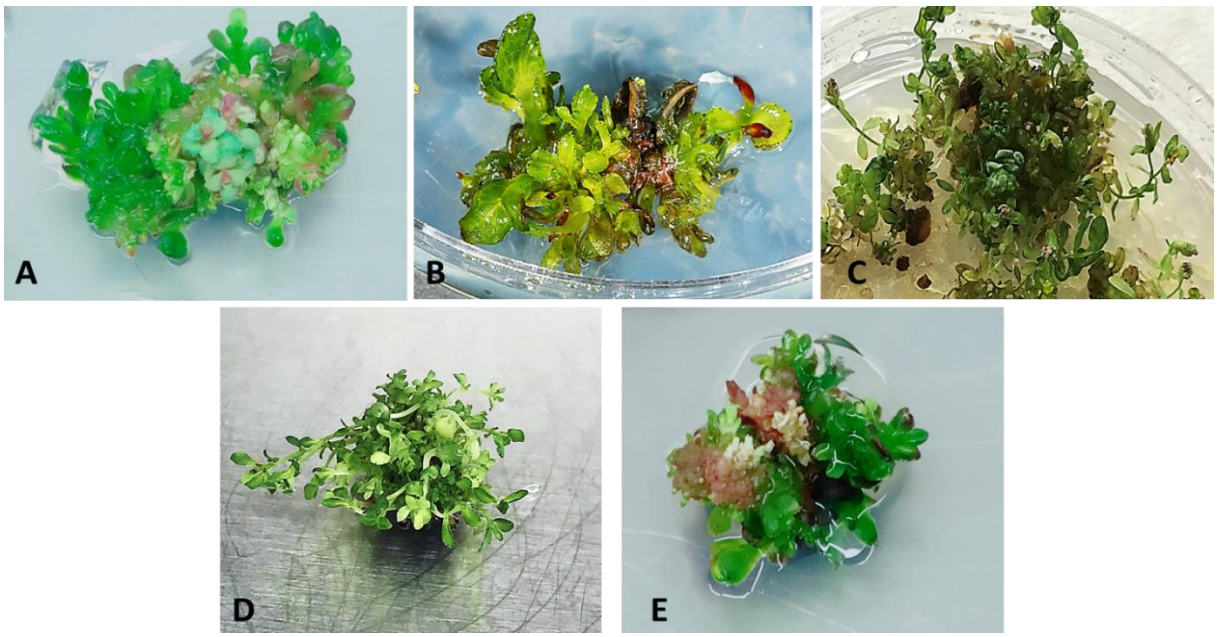

Figure 4- Development of shoot primordias and adventitious shoot regeneration of *Hypericum* species on MS medium supplemented with 1.0 mg/L BAP and 0.1 mg/L 2,4-D. (A) H. *perforatum* L. (B) H. *athoum* Boiss. & Orph. (C) H. *humifusum* L. (D) H. *leptophyllum* Hochst. (E) H. *heterophyllum* L

Comparing cytokinine types included in MS media, four species responded better to BAP in our study. High levels of BAP combined with low levels of 2,4-D comparatively induced regeneration frequency four genotypes. Recent studies have shown that many efficient adventitious callus/shoot regenerations and micropropagation methods of different *Hypericum* ssp were reported until date (Cellarova et al. 1992; Moura 1998; Pretto & Santarem 2000; Mccoy & Camper 2002; Karpinen et al. 2006; Ayan & Cirak 2006; Mulinacci et al. 2008; Karakaş et al. 2009; Coste et al. 2011; Banerjee et al. 2012; Afsharzaleh et al. 2021; Mikhovich et al. 2021; Selvakesavan & Gregory 2021; Ravindran et al. 2022). Similarly, in previous studies conducted with H. *foliosum* Aiton, H. *perforatum* L., H. *maculatum*, H. *angustifolium*, H. *hirsutum* L., and H. *triquetrifolium*, it was stated that the combined use of BAP and NAA as a cytokinin/auxin source significantly promoted adventitious shoot regeneration (Moura 1998, Pretto & Santarem 2000; Mulinacci et al. 2008; Karakaş et al. 2009; Coste et al. 2011). BAP also has critical a role in hypericin production in *Hypericum in vitro* conditions (Karakaş et al. 2009). Moreover, basal media containing high concentrations of BAP produced shoots with more branched as indicated other studies (Cellarova et al. 1992; Koperd´akova, et al. 2009). Our results also revealed that high BAP concentration and low concentration of 2,4-D promoted callus and regeneration with well-developed shoots (Figure 4).

3.2. Regeneration of Hypericum ssp. using cell suspension culture techniques

Cell suspension cultures was derived from inducing callus in leaf explants of *Hypericum* species seedlings on MS medium supplemented with 1 mg/L 2,4-D, 0.1 mg/L BAP and 7 g/L agar. Callus development and suspension cultures were induced as reported previous studies developed using leaf explants *in vitro* grown seedlings of *Hyperium* species (Bais et al. 2002; Gadzovska et al. 2007; Walker et al. 2002; Wang et al. 2015; Zubrick´a et al. 2015; Afsharzadeh et al. 2021; Ravindran et al. 2022).

These clusters were detached from the basal and transferred to cell suspension culture medium. This medium MS basal media with Gamborg's B5 vitamins containing 30 g/L sucrose, and 2 mg/L glycine, 1 mg/L 2,4-D, 0.1 mg/L KIN, and 0.1 mg/L BAP. BAP either alone or in combination with NAA is the most preferred plant growth regulators for enhanced production of secondary metabolites in *in vitro* shoot cultures of H. *perforatum* (Karppinen et al. 2007; Gadzovska et al. 2014; Kwiecień et al. 2015; Kwiecień et al. 2018). However, we added KIN and 2,4-D to suspension culture as KIN and 2,4-D promoted suitable globular calli formation and shoot regeneration for some *Hyperium* species.

The friable calli in the suspension culture began to grow within 2-4 weeks and all species formed white, healthy, friable, lightly dispersed and granular calli clusters. Percentage of volumetric callus growth of the species was fluctuated between 45-223%. The highest callus growth ratio was respectively observed in H. *perforatum* (223%) and H. *humusifum* (212%). However, H. *leptophyllum* (45%) responded the minimum callus proliferation in (Figure 5).

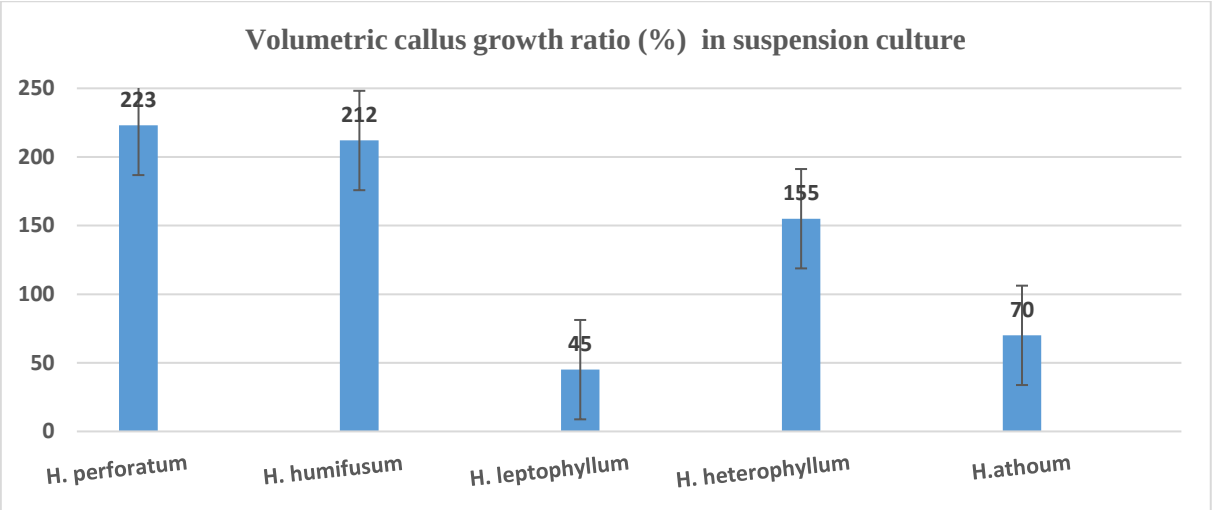

Figure 5- Volumetric callus growth ratio of *Hypericum* species on callus induction medium

The colour of cell suspension turned into different light and dark brown colour tones for each *Hypericum* species after 2-3 weeks later. We observed that phenol synthesis of the species was at the different level and death of tissue and cells (Figure 6). To provide culture conditions more stable subcultures were performed every 2 weeks. Subculturing of calli affected the formation and quality of embryonic cells as well as the sustainability of the suspension cultures. Subculturing the suspensions every 2 weeks considerably was observed to increase biomass production and number of aggregates. Homogenous cell suspensions and subculture interval provide uniform access to nutrition precursors and growth regulators and facilitate to determine critical stage of competent suspension cells (Mustafa et al. 2011; Kong et al. 2020).

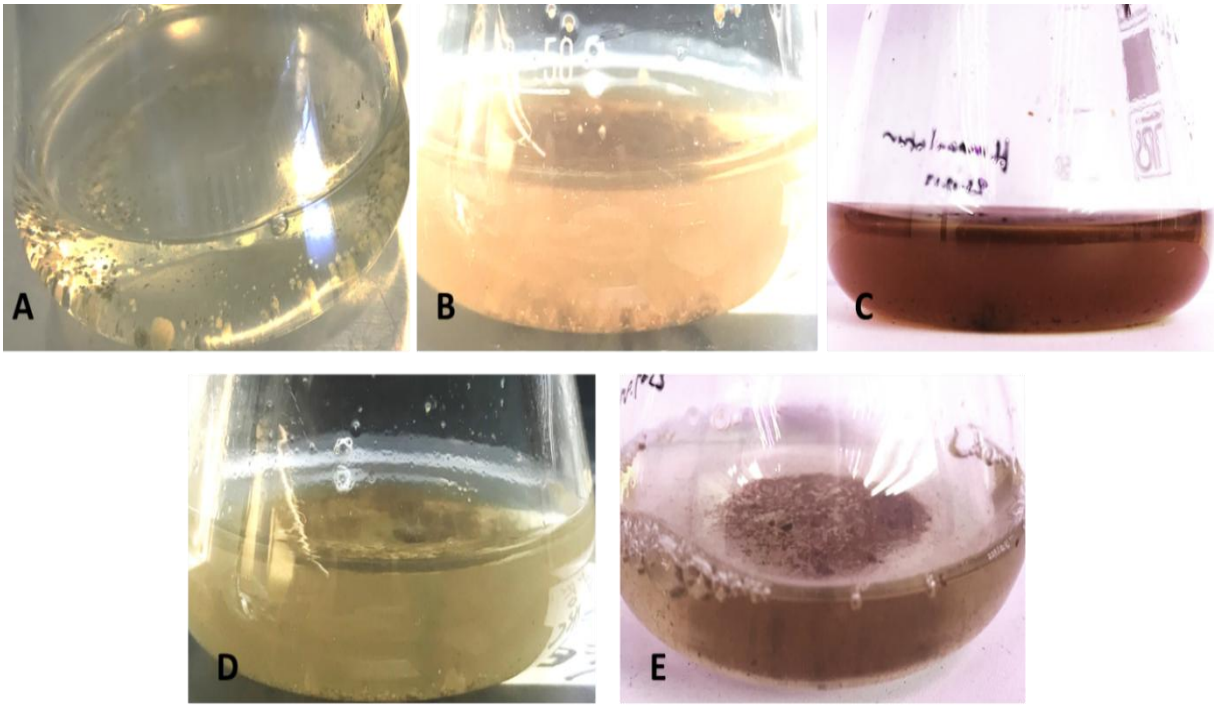

Figure 6- Suspension cell cultures of *Hypericum* species (A) H. *perforatum* (B) H. *athoum* Boiss. & Orph. (C) H. *humifusum* L. (D) H. *leptophyllum* Hochst. (E) H. *heterophyllum*

The cells isolated from calli developed in suspension culture were separatable for each species examined under a light microscope. It was observed that the cells were generally homogeneous for each species (Figure 7)

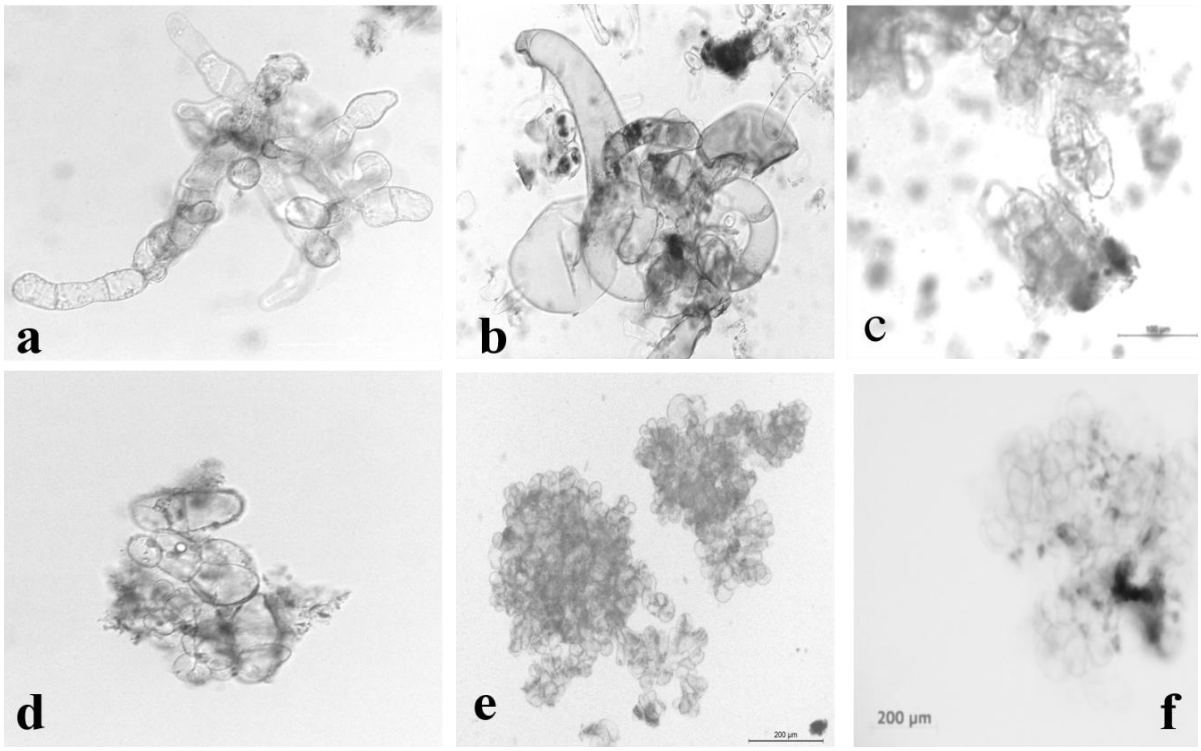

Figure 7- Light microscopic view of cells from the established suspension cultures of different *Hypericum* spp. such as (A) H. *leptophyllum* Hochst., (B) H. *humifusum* L., (C) H. *perforatum* L., H. *athom* Boiss. & Orph. and (E-F) H. *heterophyllum* L. (scale bar=200 µm)

Cell suspension cultures were plated a single globular structure after plating on agar-solidified basal media. The proembryogenic masses of suspension enlarged and formed globular, translucent, whitish separable aggregates. Protuberances were observed and turned to embryonic structures within 2-4 weeks (Figure 8). Differentiation time of somatic embryos for each species was different. Formation of somatic embryos was 1-2 weeks earlier for H. *humifusum* L., H. *heterophyllum* L. and H. *perforatum* L. species. These globular embryos further transformed into heart and cotyledonary stage. Then 2-4 weeks later, mature cotyledonary stage started, and cotyledons thickened and enlarged.

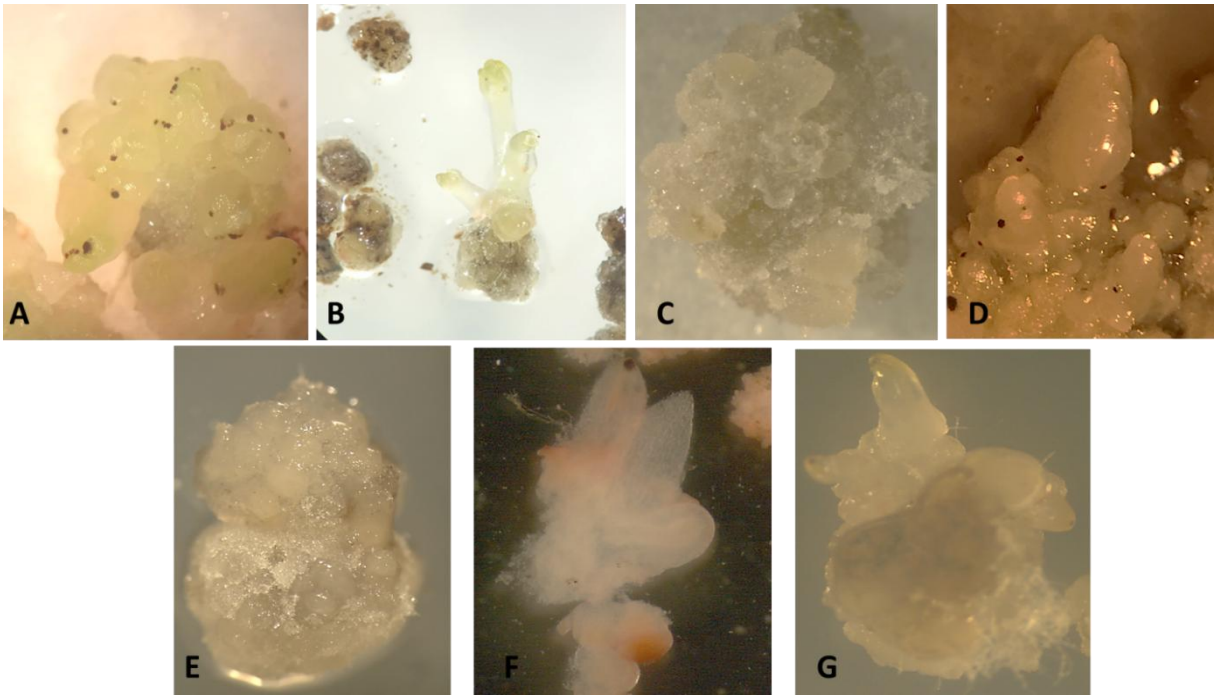

Figure 8- Embryonic callus induction and embryo proliferation after plating on agar-solidified medium in *Hypericum* species (A-B) H. *perforatum* L. *(*C) H. *athoum* Boiss. & Orph., *(*D) H. *humifusum* L., (E.) H. *heterophyllum* L. *(*F-G) H. *leptophyllum* Hochst.

Mature cotyledonary stage embryos were transferred to MS supplemented with 1 mg/L 2,4-D, 0.1 mg/L KIN, and 0.1 mg/L BAP and 7g/L agar. Germination of somatic embryos was visible for all *Hypericum* species within 3-5 weeks. The highest mean number of shoot per callus or percentage of germination of somatic embryo were respectively recorded for H. *perforatum* L. (15.37/callus), H. *leptophyllum* Hochst (6.9/callus), H. *heterophyllum* L. (9.6/callus), H. *humifusum* L. (16.8/callus) and H. *athoum* Boiss. & Orph.(11.7/callus). H. *perforatum* L. and H. *humifusum* L. showed the best shoot regeneration capacity as recorded in adventitious shoot regeneration studies (Figure 9). When germinated embryos were cultured in the same medium for more 7-8 weeks, shoot development weakened and leave began to turn yellow. Germination of mature somatic embryos decreased if they were continuously cultured on a maturation medium. All germinated somatic embryos of all species were successfully rooted. Cotyledonary stage somatic embryos germinated were rooted on MS medium containing 30 g/L sucrose with agar 7 g/L. More than 70 % of somatic embryos of all species germinated and produced thick and branched shoots. Percentage of rooting of these germinated embryos was fluctuated between average 90-100 % for each species (Figure 9). Long, mass, white and healthy roots were observed on full strength MS medium. Root growth was optimized in full strength MS medium supplemented with 0.2 mg/L IBA. Most of the embryos of all species converted to whole plants with all their organs fully developed after 2-5 weeks later on germination medium. Rooted plantlets were carried to pots including a turf and vermiculite (in a 1:1 proportion) mixture. The plants were acclimatized with 85-100 % survival rate 4-5 weeks later after transferring the greenhouse (Figure 9). H. *perforatum* and H. *humifusum* L. had also the highest survival rate (100%) as having regeneration capacity.

Recently, a few cell suspension cultures techniques of *Hyperium* ssp. have been also used for H. *androsaemum* L. (Dias et al. 2000), H. *perforatum* L. (Dias et al. 2001; Bais et al. 2002; Cui X-H et al. 2010; Gadzovska et al. 2013; Wang et al. 2015), H. *calycinum* L. (Klingauf et al. 2005), H. *triquetrifolium* (Karakaş et al. 2015), H. *androsaemum* L., H. *linariifolium* Vahl, H. *elodes* L., H. *pulchrum* L., H. *humifusum* L., H. *undulatum* Schousb. ex Willd, H. *perfoliatum* L., H. *canariense* L., H. *tomentosum* L., H. *perforatum* L. and H. *maculatum* Crantz (Selvakesavan & Gregory 2021). In our study, H. *perforatum* L., H. *leptophyllum* Hochst, H. *heterophyllum* L. H. *humifusum* L. and H. *athoum* Boiss. & Orph. were also successfully propagated using cell suspension cultures techniques.

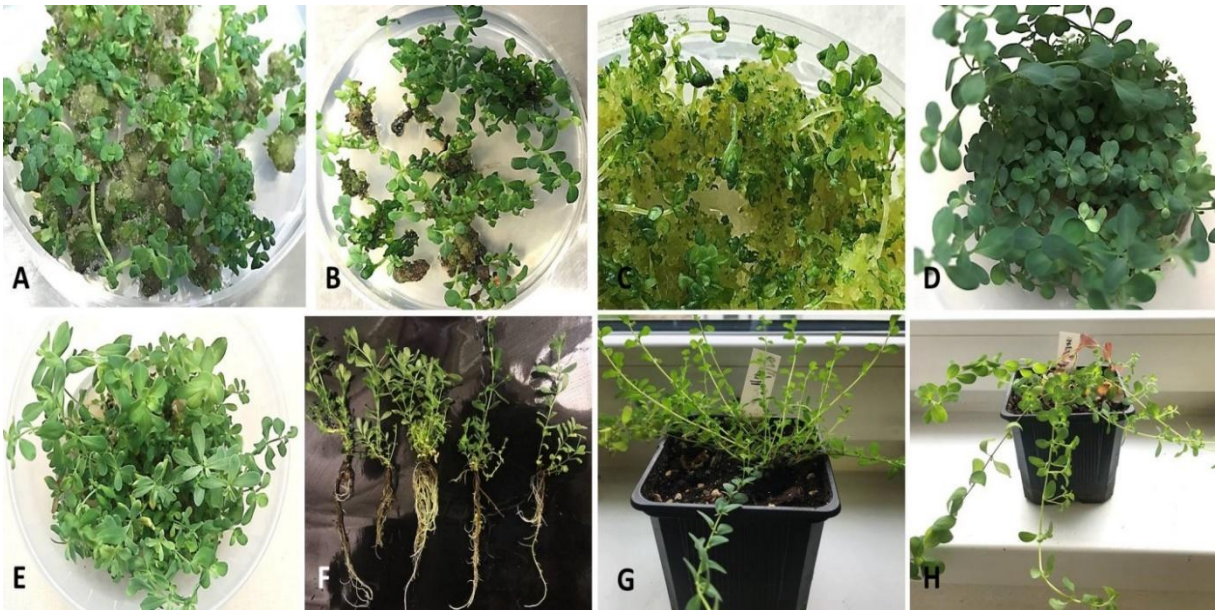

Figure 9- Germination of cell suspension-derived embryos, shoot regeneration, rooting and acclimatization of *Hypericum* species cultured suspension culture (A) H. *perforatum* L. (B) H. *humifusum* L. (C) H. *leptophyllum* Hochst, (D) H. *athoum* Boiss. & Orph (E) H. *heterophyllum* (F) Rooting of *Hypericum* spp. MS medium containing 0.2 mg/L IBA (G-H) Well-acclimatized 10-12 weeks old plantlets of H. *humifusum* and H. *perforatum* L.

4. Conclusions

Plant tissue culture methods are an important and efficient effective tool for multiplying of the species in short time, pathogen free production and providing availability of desired quantity of bioactive compounds as being in *Hypericum* species containing rich and valuable secondary metabolites. The field cultivation of *Hypericum* species may be time consuming and labour intensive and there are many biotic and abiotic obstacles that prevent high secondary metabolite yields from plants exposed to different environmental conditions (Coste et al. 2021; Tavakoli et al. 2020; Shasmita et al. 2023). Since these barriers have resulted in insufficient production of metabolites from H. *perforatum* L. we need a large-scale production system for plant and its bioactive compounds (Shasmita et al. 2023). Therefore, efficient and reliable tissue and organ culture, callus

culture, cell suspension techniques for each *Hyperium* species must be improved. In this study, it was developed high frequency regeneration protocol for five *Hypericum* species and efficient and homogenous suspension culture was established for initiation H. *perforatum* L, H. *athoum* Boiss. & Orph, H. *humifusum* L., H. *leptophyllum* Hochst, H. *heterophyllum* L. using leaf-derived calli. H. *perforatum* L. and H. *humifusum* L. had high callus and shoot regeneration capacity and positively responded to suspension cultures compared to other species.

Acknowledgements

Thankful to Inst. of Plant Gen. and Bio. Plant Sci. and Biodiv. Center, Slovak Academy of Sci. Nitra, Slovak Republic for providing facility and supporting the present research work. Distinguished thanks are due to Prof. Dr. Eva Čellárová (UPJS, Fac. of Sci. Inst. of Bio. and Eco. Dept. of Gen. Košice-Slovak Republic) for their kindly gift of *Hypericum humifusum* and *Hypericum athoum* seeds.

Funding

The authors declare that the study is funded by the Slovak Academic Information Agency / Slovak Republic.

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