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# *IN VITRO* MULTIPLICATION AND GC/MS-BASED METABOLIC PROFILES OF *CICHORIUM INTYBUS* L.

*Maria Petrova*[1], Milena Nikolova[2], Lyudmila Dimitrova[1], Margarita Dimitrova[1], Iskren Sergiev[1]*

***Address(es):***
[1] Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bldg. 21, 1113 Sofia, Bulgaria.
[2] Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bldg. 23, 1113 Sofia, Bulgaria.

*Corresponding author: marry_petrova@yahoo.com

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**ABSTRACT**

*Cichorium intybus* L. (Asteraceae) is one of the most widely used medicinal plants globally. The plant species is of great economic interest due to its high content of secondary metabolites. The present study was performed to compare the GC/MS-based metabolic profiles and total phenolic content of micropropagated and wild-growing plants. An optimized protocol for *in vitro* multiplication of *C. intybus* using stem segments from *in vitro* raised seedlings was developed. The optimum nutrient media were found to be MS medium supplemented with 1 mg/L BAP and 0.1 mg/L NAA and MS medium fortified with 1 mg/L 4PU-30 and 0.1 mg/L NAA, giving an average of 9.2±0.47 and 7.1±0.41 shoots per explant, respectively. The phenylurea cytokinin 4PU-30, first used for chicory micropropagation, effectively promoted plant regeneration and prevented hyperhydricity in *in vitro* plant tissue. Microshoots rooted successfully in half-strength MS medium free of plant growth regulators. All plants were hardened and survived transfer to *ex vitro* conditions. No differences were found between the GC/MS-based metabolic profiles of the wild-growing plants and those multiplied *in vitro* and acclimated to controlled field conditions. A quantitative difference was obtained in some individual metabolites: esculetin and quinic acid were higher in samples of *in vitro* obtained plants, while chlorogenic acid was more abundant in samples of wild-growing plants.

**Keywords:** micropropagation, callus, total phenols, metabolites, medicinal plant

## INTRODUCTION

*Cichorium intybus* L. (Asteraceae) has a long history of use as a medicinal plant dating from ancient times. The species commonly known as chicory is one of the most popular medicinal plants on the global market and is also applied as a coffee substitute, vegetable crop and animal forage. Chicory is a unique plant species extremely rich in secondary metabolites – alkaloids, coumarins, sesquiterpene lactones, flavonoids, terpenoids, steroids, volatile compounds, phenolic acids, organic acids, caffeic acid derivatives (**Nandagopal and Kumari, 2007; Aisa *et al.* 2020**). The plant has a number of activities – antimicrobial, anthelmintic, antimalarial, hepatoprotective, antidiabetic, gastroprotective, anti-inflammatory, antioxidant, tumor-inhibitory etc. (**Street *et al.*, 2013**). The high protein content, carbohydrates, minerals, and vitamins make it a particularly valuable crop both for humans and animals (**Aldahak *et al.*, 2021**). The first attempts to grow *C. intybus* began 4000 years ago (**Wang and Cui, 2011**). The conventional method of vegetative propagation is limited by variations in abiotic and biotic environmental conditions. Micropropagation is a good alternative to provide quality stock plants, preserve valuable genotypes and forms, and overcome the genetic and phenotypic variability of wild-growing plants (**Debnath *et al.*, 2006**). In the literature, there are a number of studies concerning the micropropagation of *C. intybus*. Most authors are unanimous that organogenesis in chicory passes through the callus phase and the frequency of direct organogenesis is lower (**Abdin and Ilah, 2007**; **Dakshayini *et al.*, 2016**; **Doliński and Olek**, **2013**). The commonly used cytokinins for shoot development *in vitro* of *C. intybus* are $N^6$-benzylaminopurine (BAP), kinetin, zeatin or thidiazuron (TDZ) usually in combination with some of the following auxins: α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA) or indole-3-butyric acid (IBA) (**Doliński and Olek**, **2013; Maroufi *et al.* 2012; Shahin *et al.* 2015; Dakshayini *et al.*, 2016)**. Plants growing in wild populations vary in terms of morphology and content of biologically active substances (**Doliński and Olek, 2013**). *In vitro* micropropagation enables large-scale production of homogenous, disease-free plants producing phytochemicals with consistent yield and quality (**Espinosa-Leal *et al.*, 2018**). It is of interest to determine whether changes have occurred in the chemical composition of the micropropagated plants compared to the initial plants collected from the wild population. Often, the conditions of *in vitro* cultivation (high relative humidity, low ventilation rate, high concentrations of growth regulators, etc.) act as stress factors, causing changes in the secondary metabolism of plants. In the literature, knowledge of the chemical composition of micropropagated *C. intybus* plants is rather scarce. It was found that the combination and concentrations of plant growth regulators (PGRs) used in Gamborg's (B5) and Murashige and Skoog (MS) media were not only essential for the formation of callus, shoots, and roots but also caused a change in the amounts of some phenolic components (caftaric, chicoric, and chlorogenic acids and esculin) (**Abas *et al.*, 2023**). The high content of inulin and esculin from extracts of leaves and roots was detected in *in vitro* regenerated plants through HPLC (**Rehman *et al.*, 2003; Kumari *et al.*, 2007; Ohadi Rafsanjani *et al.*, 2011**). Most chicory phytochemical investigations are conducted using HPLC, but GC/MS is used when the emphasis is on non-polar compounds. Studies investigating the GC/MS-based metabolite profiles of *C. intybus* mainly refer to wild plants. Among the 78 different compounds identified in the methanol extract of wild chicory leaves, the main phytochemical constituents were phytol and stigmast-5-en-3-ol (**Malik *et al.*, 2017**). The 64 common metabolites were recognized from the leaves of 7 chicory specimens collected from different altitudes, with methyl commate B, gamma sitosterol, and 9, 12, 15-octadecatrienoic acid predominant (**Malik *et al.*, 2022**). The objective of this study was to develop an effective micropropagation protocol for *C. intybus*, and to compare the total phenolic content and metabolic profiles of micropropagated and wild-growing plants.

## MATERIALS AND METHODS

### Seed sterilization

The seeds were collected from plants growing in a wild population on the Vitosha Mountain, near the village of Bistritsa, Bulgaria. Seeds were washed with tap water and a detergent. Soaking seeds in 70% ethanol for 2 min followed by 0.1% mercuric chloride for 20 min was used for seed sterilization. Afterwards, three-fold rinses in autoclaved distilled water were performed. The sterilized seeds were cultured on **Murashige and Skoog, 1962** (MS) medium free of plant growth regulators (PGRs). Fifty seeds were used to test the germination *in vitro* in two replications.

### Shoot multiplication

Axenic explants (stem segments excised from two-month-old *in vitro* germinated seedlings) were used for the initiation of *in vitro* culture. MS media supplemented with different types of cytokinins $N^6$-benzylaminopurine (BAP), kinetin (Kn), $N_1$-(2-chloro-4-pyridyl)-$N_2$-phenylurea (4PU-30), zeatin (Z) or $N^6$[2-isopentenyl]-adenine (2-iP) at concentration 1 mg/L, combined with the auxin α-naphthalene acetic acid (NAA) at concentration 0.1 mg/L were applied for induction of shoot multiplication. In addition, two nutrient media abbreviated $B_{0.5}N_{0.1}$ and $Kn_{0.5}N_{0.1}$,

containing 0.5 mg/L of cytokinin and the same amount of auxin NAA (0.1 mg/L) were tested. A control medium without PGRs was also considered. All media contained 3% table sugar and were solidified with 0.7% agar-agar (bacteriological) (w/v). The pH of all nutrient media was adjusted to 5.8 with 1N NaOH or 1N HCl. For sterilization, the media were autoclaved at 121 °C for 20 minutes. After five weeks of incubation, the frequency of shoot proliferation (measured as the percentage of explants forming shoots), the average number of shoots per explant (>1 cm length), and the mean shoot length were all calculated. The percentages of explants with callusogenesis and rooting observed in some treatments were also estimated. The micropropagation experiments were carried out using glass tubes with 10 ml of nutrient medium per tube. Each experimental treatment included 40 explants, in duplicate. *In vitro* cultures were maintained in a growth room at a temperature of 22 ± 2 °C under a 16 h photoperiod with a light intensity of 40 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent tubes.

***In vitro* rooting and acclimatization**

The multiple shoot clumps were separated and elongated shoots were cultured individually on MS half-strength medium without PGRs, containing 2% table sugar and 0.7% agar. The percentage of rooted shoots, the mean number of roots per explant, and the mean length of roots were recorded after 4 weeks of incubation. The experiment for *in vitro* rooting included 40 explants in duplicate. Plantlets with well-developed roots were removed from the nutrient medium, washed gently under running tap water and planted into plastic pots (8 cm in diameter) containing a substrate mixture of soil, peat, perlite, and sand in the ratio 2:1:1:1 (v/v/v/v). The pots were covered with clear plastic boxes to maintain high relative humidity within 2 weeks and were cultured at 24±1 °C temperature. The survival rate was recorded 5 weeks after adaptation under *ex vitro* conditions. The plants were then transferred to field plots.

**Plant material extraction**

*Diethyl ether extracts.* Samples of air-dried aerial parts of the *in vitro* obtained, outdoor acclimatized plants and wild-growing plants (100 mg) were extracted with diethyl ether for 24 h. The extracts were filtered into glass vial and were dried at ambient temperature.
*Methanolic extracts.* Air dried aerial parts of the studied samples (100 mg) were extracted with methanol for 24 h. The extracts were filtered into glass vial and were dried at ambient temperature. 50 µl of 3,5-dichloro-4-hydroxybenzoic acid (1 mg/mL) were placed in the beginning of the extraction procedure as internal standard.
*Methanol insoluble alkaline hydrolyzable phenolic acids fractions.* Plant material remaining after extraction with methanol was hydrolyzed by 2M NaOH for 4 h at room temperature. After acidification to pH 1-2 with conc. HCl, the phenolic compounds were extracted with EtOAc which was dried with anhydrous $Na_2SO_4$ and evaporated to obtain alkaline hydrosable phenolic acids. In the beginning of the extraction procedure 50 µl of 3,4-dichloro-4-hydroxybenzoic acid (1 mg/ml) were added as internal standard

**Derivatization**

100 µl pyridine and 100 µl of N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) were added to the dried samples of the extracts and the fractions were heated at 70 °C for 2 h. After cooling, 300 ml of chloroform were added, and the samples were analyzed by GC/MS.

**GC/MS analysis**

GC/MS analysis of extracts and fractions was recorded on a Thermo Scientific Focus GC coupled with Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. A DB-5MS column (30 m x 0.25 mm x 0.25 µm) was used. The conditions of the analysis were described by **Berkov *et al.*, 2021**. The GC-MS spectra of the compounds in the extracts were deconvoluted by AMDIS 2.64 software (NIST, National Institute of Standardization and Technology, Gaithersburg, MD) before their comparison with those of standard compounds and NIST spectra library. All experiments were repeated three times.

**Determination of the total phenolic content**

The total phenolic content of methanol extracts was determined using Folin-Ciocalteu reagent and gallic acid as standard. Methanolic extracts were diluted to a concentration of 2 mg/ml, and aliquots of 0.200 ml were mixed with 2 ml of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and 1.8 ml of $Na_2CO_3$ (7.5%). After 1 h at room temperature, the absorbance of the samples was measured at 765 nm on a spectrophotometer versus a blank sample. Total phenols were determined as gallic acid equivalents (mg GA) per gram of extract.

**Statistical analysis**

Data were subjected to one-way ANOVA analysis of variance for comparison of means, and significant differences were calculated according to the Fisher LSD test at the 5% level using a statistical software package (Statigraphics Plus, version 5.1 for Windows). Data were reported as means ± standard error.

**RESULTS**

***In vitro* culture initiation**

The sterilization procedure was effective, and all planted seeds (100 %) were contamination-free. Among them 60% germinated on MS medium free of PGRs. All *in vitro* seedlings survived and were grown on MS medium without PGRs within 4 weeks to generate plant material for microropagation studies.

**Shoot multiplication**

All explants cultured on MS media supplemented with different PGRs, with the exception of the control (PGRs free MS medium) formed calli at the cut ends within two weeks of incubation. The efficiency of callus induction and shoot regeneration depended on the applied plant growth regulators (Table 1). Callus and bud formation were more pronounced on the nutrient media with a higher concentration of cytokinin (1 mg/L). The highest percentage of organogenic callus was observed on $B_1N_{0.1}$ medium followed by 4PU-$30_1N_{0.1}$ medium, 90 and 80% respectively. Induction of the shoots via callus started after 7-14 days of cultivation on the tested media. Multiple shoot development was observed at the end of the $5^{th}$ week. The highest response to shoot multiplication was achieved on MS medium supplemented with 1 mg/L BAP and 0.1 mg/L NAA, yielding 9.2±0.47 shoots per explant (Table 1; Figure 1a). Some explants developed up to 25 shoots. This PGR combination in MS medium provoked the highest shoot organogenesis (100% efficiency). In addition to well-formed shoots, numerous buds were counted. However, 10% of the developed shoots showed hyperhydricity. The decrease of BAP concentration to 0.5 mg/L in the nutrient medium caused a reduction in the percentage of explants with organogenic callus as well as the mean number of shoots per explant. The second most effective medium was 4PU-$30_1N_{0.1}$ containing poorly studied phenylurea cytokinin (4PU-30) and NAA where pale-yellow and greenish calli producing numerous shoots (7.1±0.41 per explant) were noticed (Figure 1 b). Multiple shoot development was also recorded on MS medium supplemented with 1 mg/L kinetin and 0.1 mg/L NAA. The presence of 2-iP, zeatin and kinetin in the nutrient media induced rhizogenesis in about 25-50% of the shoots (Table 1). The shoots developed on the control medium without any PGRs exhibited the highest percentage of rooting (80% rooted) and reached the maximum length (8.8±0.44 cm). Repeated sub cultivation is an efficient way for obtaining new shoots. In the next sub cultivation, the frequency of callusogenesis was lower and shoots developed directly from the explant (Figure 1c and d).

**Table 1** Effect of the PGRs on the micropropagation of *Cichorium intybus*

| **Nutrient medium** | **Organogenic callus**, % | **Shoot formation**, % | **Shoots/explant** Mean ±SE | **Shoot length**, cm | **Rooted shoots** % |
|---|---|---|---|---|---|
| MS | nd | 40 | 1.6±0.15$^a$ | 8.80±0.44$^d$ | 80% |
| $B_1N_{0.1}$ | 90 | 100 | 9.2±0.47$^f$ | 3.15±0.33$^a$ | nd |
| 4PU-$30_1N_{0.1}$ | 80 | 95 | 7.1±0.41$^e$ | 3.95±0.43$^a$ | nd |
| $Kn_1N_{0.1}$ | 70 | 90 | 5.2±0.20$^d$ | 3.35±0.18$^a$ | 30% |
| $Z_1$ $N_{0.1}$ | 40 | 80 | 3.1±0.32$^b$ | 5.25±0.47$^b$ | 25% |
| 2-$iP_1N_{0.1}$ | 30 | 60 | 2.7±0.16$^b$ | 6.60±0.42$^c$ | 50% |
| $B_{0.5}N_{0.1}$ | 60 | 90 | 6.0±0.34$^d$ | 4.00±0.22$^a$ | nd |
| $Kn_{0.5}N_{0.1}$ | 10 | 90 | 4.3±0.33$^c$ | 5.05±0.37$^b$ | 50% |
| LSD | | | 0.89 | 1.05 | |

**Legend:** The data are presented as means of 40 shoots per medium variant ± standard error. Different letters indicate significant differences assessed by Fisher LSD test (5%) after performing one way ANOVA analysis; nd – not developed

**Root induction and acclimatization**

Rhizogenesis was induced within the first 5–7 days of culture on half strength MS medium. All shoots rooted, with an average of 6–8 roots per shoot (Figure 1e). The acclimatization of *in vitro* obtained plants to outdoor conditions is one of the most important stages in the micropropagation protocol. The soil mixture, consisting of soil, peat, perlite and sand in a 2:1:1:1 ratio was found to be optimum for the hardening of the plants. The microplants of *C. intybus* were successfully adapted to *ex vitro* conditions (Figure 1f). The survival rate reached 100%. The plantlets were transferred to the experimental field plot of the Institute of Plant Physiology and Genetics (Sofia, Bulgaria) during the autumn. All plants acclimatized successfully and bloomed the next year.

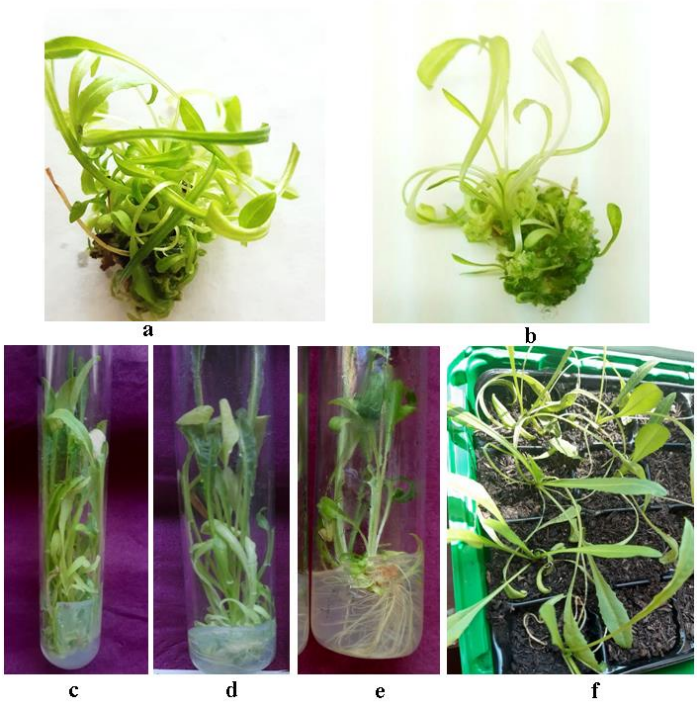


**Figure 1** *In vitro* cultivation of *Cichorium intybus*. Shoot regeneration on media: a) $B_1N_{0.1}$ and b) 4PU-$30_1N_{0.1}$; Multiplied plantlets on media: c) $B_1N_{0.1}$ and d) 4PU-$30_1N_{0.1}$ in the second subculture; e) *In vitro* rooting on half strength MS medium; f) *Ex vitro* adapted plants.

**Metabolic analysis**

Metabolites of diethyl ether and methanolic extracts of *in vitro* and wild-growing plants were identified by GC/MS. The results are presented in Table 2 and Table 3. Fatty acids, sterols and triterpenes were determined as the most abundant compounds in the diethyl ether extracts (Table 2; Figure 2). Hexadecanoic acid was found as the main component. Amyrin and triterpenes were also presented as dominant compounds. In diethyl ether extracts, phenolic and organic acids, monosaccharides, fatty and sugar alcohols were also determined but in small quantities. No differences in the metabolic profiles of the plants from studied samples were detected. Esculetin and lupeol predominated in the *in vitro* samples. In the methanolic extracts, polar compounds were found as the most abundant, such as sugars (fructose, glucose, sucrose), polyols (*myo*-inositol) (Table 3; Figure 3). Nine phenolic acids were determined. Quinic and caffeic acids were the most abundant. Chlorogenic and ferulic acids were presented in significant amounts as well. Organic and fatty acids, sterols and triterpenes were also found. No differences in the profiles between *in vitro* and wild-growing plants of the species were found. Eight phenolic acids were identified after alkaline hydrolysis on the residue plant material after methanolic extraction. The results are presented in Table 3; Figure 4. Caffeic acid *trans* and 4-hydroxycinnamic acids were determined as the main ones. No differences in the phenolic acid quality composition between the studied samples from *in vitro* and wild-growing plants were found. Some quantitative differences in the content of individual metabolites were observed. Quinic acid, *trans* caffeic acid and malic acid were more abundant in the *in vitro* samples, while chlorogenic acid, myo-inositol and sucrose were higher in the wild plant samples.

**Table 2** Identified metabolites in diethyl ether extracts of the studied samples.

| Metabolites | RI | Studied samples | |
|---|---|---|---|
| | | *In vitro* plants; Area [%] | Wild plants; Area [%] |
| Hexanoic acid | 1071 | 1.4 | 1.7 |
| Octanoic acid | 1265 | 1.1 | 1.1 |
| Dodecanoic acid | 1521 | 0.2 | 0.2 |
| 4(p)-Hydroxybenzoic acid | 1635 | 0.3 | 0.1 |
| Fructose 1 | 1800 | 1.8 | 2.8 |
| Fructose 2 | 1837 | 0.3 | 0.1 |
| Tetradecanoic acid | 1850 | 2.8 | 5.4 |
| Glucose | 1882 | 0.4 | 0.4 |
| Hexadecanoic acid (*Palmitic acid*) | 2041 | 13.3 | 11.7 |
| *myo*-Inositol | 2080 | 0.5 | 0.6 |
| 6,7-Dihydroxycoumarin (*Esculetin*) | 2125 | 8.9 | 4.4 |
| Octadecanol | 2157 | 0.1 | 0.03 |
| 9,12 Octadecadienoic acid (*Linoleic acid*) | 2201 | 1.8 | 0.2 |
| 9-Octadecenoic acid | 2227 | 0.8 | 0.2 |
| Octadecanoic acid (*Stearic acid*) | 2239 | 1.4 | 1.4 |
| Sucrose | 2628 | 1.3 | 1.7 |
| Tetracosanoic acid | 2826 | 0.3 | 0.5 |
| Campestrol | 3239 | 0.5 | 0.4 |
| Stigmasterol | 3319 | 1.7 | 1.2 |
| β-Amyrin | 3335 | 3.7 | 1.1 |
| α-Amyrin | 3382 | 4.5 | 3.6 |
| Lupeol | 3434 | 10.4 | 6.0 |
| Triterpen 1 (*lypeol type*) | 3525 | 5.3 | 6.8 |
| Triterpen 2 (*lupeol type*) | 3530 | 0.7 | 1.4 |

**Legend:** Area [%] The quantities of the compounds were expressed as the percentage area of the total peaks' area of the chromatogram.

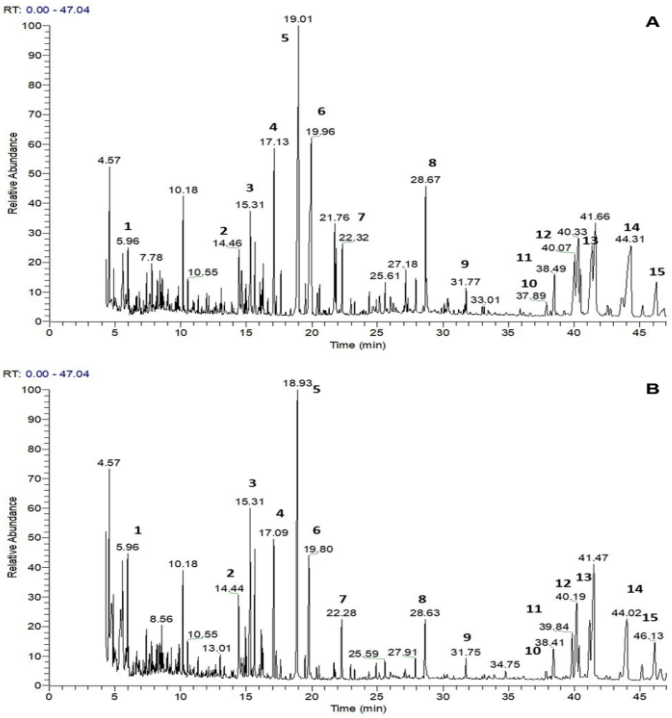


**Figure 2** GC/MS chromatograms of diethyl ether extracts of *in vitro* (A) and wild-growing plants(B) with main peaks
1.Hexanoic acid; 2. Fructose; 3. Tetradecanoic acid; 4. *myo*-Inositol 5. Hexadecanoic acid; 6. Esculetin; 7. Octadecanoic acid; 8. Sucrose 9. Tetracosanoic acid; 10. Stigmasterol 11. Sitosterol; 12. β-Amyrin; 13.α-Amyrin 14. Lupeol 15. Triterpene

**Table 3** Identified compounds in the methanolic extract and ethyl acetate fraction of the studied samples.

| Compounds | RI | MeE *in vitro plants* | MeE *wild plants* | Fraction *in vitro plants* | Fraction *wild plants* |
|---|---|---|---|---|---|
| | | Response ratio* | | | |
| **Phenolic acids** | | | | | |
| 4(p)-Hydroxybenzoic acid | 1635 | 0.3 | 0.4 | 1.4 | 2.2 |
| Vanillic acid | 1776 | 0.4 | 0.4 | 0.5 | 0.3 |
| *cis* 4(p)-Hydroxycinnamic acid | 1783 | | | 1.0 | 5.2 |
| Protocatechuic acid | 1813 | 0.6 | 0.6 | | |
| Quinic acid | 1843 | 91.4 | 56 | | |
| *cis* Ferulic acid | 1863 | | | 0.8 | 2.5 |
| Syringic acid | 1888 | 0.3 | 0.4 | | |
| *trans* 4(p)-Hydroxycinnamic acid | 1948 | 8.1 | 6.1 | 15.5 | 20.1 |
| *cis* Caffeic acid | 1984 | | | 0.8 | 0.9 |
| *trans* Ferulic acid | 2086 | 10.8 | 10.5 | 5.6 | 14 |
| *trans* Caffeic acid | 2141 | 56 | 34.6 | 69.2 | 25.1 |
| Chlorogenic acid | 3100 | 15.1 | 47.5 | | |
| **Organic acids** | | | | | |
| Succinic acid | 1310 | 50.5 | 54.8 | | |
| Glyceric acid | 1340 | 11.4 | 10.3 | | |
| Malic acid | 1488 | 272.6 | 38.4 | | |
| **Polyols** | | | | | |
| Erythritol | 1700 | 4.2 | 16.8 | | |
| *myo*-Inositol | 2090 | 1518.4 | 2245.2 | | |
| **Sugars** | | | | | |
| Fructose 1 | 1793 | 6.6 | 26.1 | | |
| Fructose 2 | 1803 | 16.2 | 30.9 | | |
| Glucose | 1889 | 4.3 | 2.6 | | |
| Galactose | 1915 | 11.5 | 51.7 | | |
| Sucrose | 2628 | 528.7 | 1741.1 | | |
| **Fatty alcohols** | | | | | |
| Glycerol | 1260 | 1233 | 843 | | |
| Tetradecanol | 1759 | 0.5 | 0.3 | | |
| **Fatty acids** | | | | | |
| Hexadecanoic acid | 1925 | 224.5 | 279.7 | | |
| Octadecanoic acid | 2132 | 52.8 | 73.1 | | |
| Octadecadienolic acid | 2201 | 967 | 300 | | |
| **Coumarins** | | | | | |
| 6,7-Dihydroxycoumarin (*Esculetin*) | 2125 | 2.4 | 1.4 | | |
| **Sterols and Triterpenes** | | | | | |
| Campestrol | 3239 | 2.8 | 1.4 | | |
| Stigmasterol | 3319 | 19.1 | 13.3 | | |
| β-Sitosterol | 3335 | 36.5 | 31.4 | | |
| β-Amyrin | 3366 | 16.1 | 21.2 | | |
| α-Amyrin | 3382 | 5.1 | 5.4 | | |
| Lupeol | 3434 | 34.4 | 71.1 | | |
| Triterpene (*lupeol type*) | 3525 | 40 | 38.9 | | |

**Legend:** MeE-methanolic extract; *The response ratios represents peak area ratios using 3,5-dichloro-4-hydroxybenzoic acid (50 µg) as quantitative internal standard.

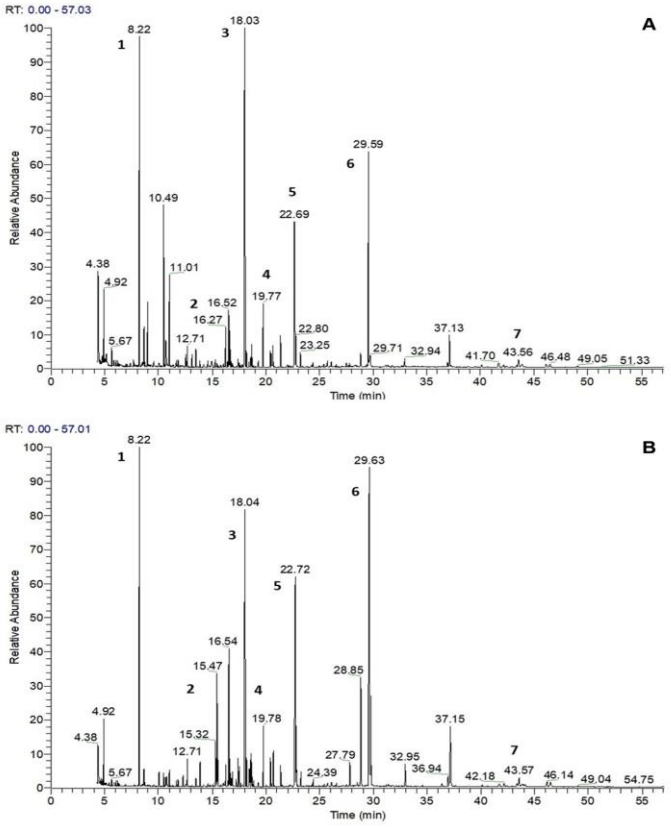


**Figure 3** GC/MS chromatograms of methanolic extracts of *in vitro* (A) and wild-growing plants (B) with main peaks 1. Glycerol; 2. Fructose; 3. Myo-Inositol; 4. Hexadecanoic acid; 5. Octadecadienoic acid; 6. Sucrose; 7. Lupeol

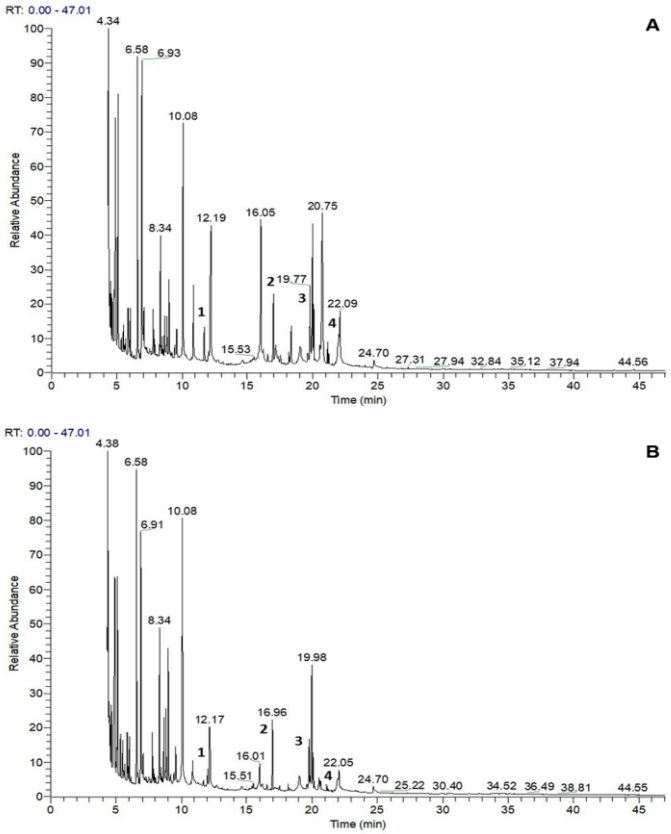


**Figure 4** GC/MS chromatograms of phenolic fractions of *in vitro* (A) and wild-growing plants (B) with main peaks 1. 4-Hydroxybenzoic acid, 2. Hydroxycinnamic acid; 3. Ferulic acid; 4. Caffeic acid

**Total phenolic content**

The total phenolic content of the studied samples was evaluated by spectrophotometric assay. No significant differences between the samples from wild and in *vitro* grown plants were detected. The established values for *in vitro* and wild-growing *in vivo* plants were 28.6±2.5 and 30.3±4.3 mg GAE/g extract, respectively.

## DISCUSSION

*Cichorium intybus* L. is one of the most important medicinal plants cultivated throughout the world. The commercial importance of *C. intybus* has led to attempts to develop alternative systems for its reproduction. Biotechnological tools, especially plant cell tissue and organ culture, provide sustainable, economical and viable production of secondary metabolites (**Chandran *et al.*, 2020**). In the current study, the reproducible protocol for *in vitro* multiplication and evaluation of the content of secondary metabolites of the micropropagated and wild-growing plants of *C. intybus* was reported. The growth of chicory in *in vitro* conditions is influenced by the genotype, initial explants, and exogenous PGR supplementation (**Doliński and Olek**, **2013; Maroufi *et al.*, 2012; Dakshayini *et al.*, 2016)**. Usually cotyledons, leaves, hypocotyls, floral stems and roots are used as explant sources for *in vitro* culture establishment of *C. intybus*. The explants are capable of developing organogenic calli which give rise to new shoots. Young tissue has been found to be more susceptible to callus and bud formation than older tissue. Our data showed that the nodal segments from young two-month-old *in vitro* germinated seedlings were suitable for *in vitro* culture initiation. There is only one report in the available literature on the use of stem explants (**Abdin and Ilah, 2007**). However, the authors observed regeneration via somatic embryogenesis. In our study on the cut ends of explants, friable pale-yellow, whitish-green callus was developed. The callus starts to differentiate, and organogenesis efficiency depends on the PGRs applied to the nutrient medium. It was previously found that NAA was the most effective auxin for callus induction (**Velayutham *et al***., **2006**). In the present study, the combination of BAP and NAA was optimal for chicory shoot organogenesis and multiplication. The other authors were not unanimous regarding the composition of PGRs in the MS nutrient medium for regeneration and shoot induction of *C. intybus*. High multiplication rate was achieved on MS supplemented with 0.5 mg $dm^{-3}$ IAA and 4 mg $dm^{-3}$ 2iP (**Doliński and Olek, 2013**), 4 µM BAP and 1 µM IAA (**Velayutham *et al.*, 2006**), 2 mg/L kinetin and 0.5 mg/L IBA (**Dakshayini *et al.*, 2016**), 0.5 mg/L IAA and 0.5 mg/L BAP (**Maroufi *et al.*, 2012**), 5µM BA and 3µM TDZ (**Shahin *et al.*, 2015**). Most authors applied a two-step system for obtaining shoot organogenesis – cultivation on medium for callus induction and sub-cultivation on medium for shoot regeneration. **Hamid *et al.*** (**2010**) used the same medium for callus formation and shoot multiplication in conformation with our findings. The second effective medium for shoot multiplication was found to be MS augmented with 1 mg/L 4PU-30 and 0.1 mg/L NAA. The cytokinin 4PU-30 was applied for the first time to test the possibility of micropropagation of chicory. It was previously found that 4PU-30 was more effective for multiplication of *Mentha pipreita* than BAP and did not alter the essential oil composition of the microplants compared to control (**Ivanova and Iliev**, **1993**, **Ivanova *et al.*, 1996**). In carnation, this phenylurea cytokinin increased the multiplication coefficient, growth, peroxidase, and superoxide dismutase activities (**Genkov and Ivanova, 1995**; **Goltsev *et al.*, 2001**). 4PU-30 similarly to other adenine derived cytokinins (BAP, kinetin, 2-iP, zeatin), has the potential to promote callusogenesis, regeneration and production of ethylene (**Genkov and Ivanova**, **1995**, **Goltsev *et al.*, 2001**). In this study, the results of the application of the cytokinins BAP and 4PU-30 are comparable despite the higher multiplication rate on BAP containing medium, due to hyperhydracity, which was observed in some explants (10%) derived from $B_1N_{0.1}$. Hyperhydricity is a physiological disorder and is a common phenomenon in *in vitro* culture, affecting growth and obtainment of healthy and vigorous microplants (**Kevers *et al.*, 2004)**. It was previously established that thidiazuron, the other phenylurea derived cytokinn, also effectively induced regeneration and multiplication of *C. intybus* (**Ohadi Rafsanjani *et al.*, 2011**).

MS medium containing half strength of salts and vitamins was successfully used for rhizogenezis and 100% of the shoots rooted. Other authors used the three most applied auxins for *in vitro* rooting – IAA, IBA, NAA. Among them, IBA gave the best results (**Rehman *et al*. 2003; Velayutham *et al.*, 2006; Nandagopal and Kumari, 2007**). We did not use auxins in our protocol, and a low-cost half-strength MS medium free of PGRs was found to be optimal for *in viro* rooting. The cost of media composition was also reduced by using table sugar instead of laboratory grade sucrose. The chicory microplants were easily adapted to *ex vitro* conditions (100% survival rate). However, the efficiency of acclimation depends on the applied methodology (soil mixture substrate and culture conditions) (**Velayutham *et al.*, 2006**; **Yucesan *et al.*, 2007**; **Doliński and Olek**, **2013**). From 56 to 100 % of plants were hardened and survived the transfer to outdoor conditions according to the literature data.

Although *C. intybus* is widespread in nature, the secondary metabolic profiles show variation as a response to adaptation to diverse abiotic and biotic stresses (**Shilpa and Lakshmi, 2019**). It was found that the total quantity of phenolic compounds and flavonoids increased in chicory plants growing on saline soil (**Zlatić and Stanković, 2017**). Plant tissue cultures have been used for mass cloning of valuable medicinal plants and represent a major platform for secondary metabolite production under constant conditions (**Chandran *et al.*, 2020**). The reports on the accumulation of biologically active compounds in chicory shoots and roots in *in vitro* cultures are limited. Auxin, cytokinin, and $GA_3$ treatment influenced coumarin production in hairy root cultures of *C. intybus* (**Bais *et al.*, 2001**). The amount of esculin and inulin from extracts of leaves and roots of *in vitro* developed *C. intybus* plants was higher compared to normally grown plants (**Rehman *et al*, 2003; Kumari *et al.*, 2007; Ohadi Rafsanjani *et al.*, 2011**). In the present study, the applied *in vitro* propagation method had an effect on the accumulation of individual compounds. Esculetin was the most abundant in extracts of aerial parts of *in vitro* plants, in accordance with the results reported by **Ohadi Rafsanjani *et al.* (2011) and Abas *et al.* (2023)**. However, the metabolic profiles of *in vitro* and *in vivo* plants were the same. In previous studies, it was established that secondary metabolites in micropropagated plants showed a slight increase compared to conventionally propagated plants due to different PGRs applied during *in vitro* cultivation (**Chavan *et al.*, 2014**; **Zahid, *et al.*, 2021**). In addition, multiplied plantlets are exposed to stressful environments during their transfer from *in vitro* to *ex vitro* conditions (**Min *et al.*, 2017**).

## CONCLUSION

The present study provides an efficient and reproducible *in vitro* protocol for the rapid multiplication of the medicinal plant *C. intybus*. The development of multiple shoots with good quality was achieved on MS medium supplemented with a combination of the rarely used phenylurea cytokinin 4PU-30 and auxin NAA. The *in vitro* shoots were spontaneously rooted on half-strength MS medium without plant growth regulators. The cost of the current *in vitro* propagation protocol was reduced by using table sugar instead of laboratory-grade sucrose. Plantlets grown *in vitro* had a high survival rate during *ex vitro* adaptation and were successfully acclimatized to the experimental field. The GC/MS analysis showed no differences between wild-growing and *in vitro* propagated plants in terms of their metabolic profiles. Some quantitative differences in the content of individual metabolites were observed between the studied samples. Esculetin, quinic acid, *trans* caffeic acid and malic acid were more abundant in the *in vitro* plants, while chlorogenic acid, myo-inositol and sucrose were higher in the wild-growing plants. The results revealed no significant differences in total phenolic content between the *in vitro* obtained and wild-growing plants. The developed *in vitro* protocol can be of great benefit for the commercial propagation of chicory and provide a promising source of valuable metabolites.

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