# Content of pyrrolizidine alkaloids (senecionine and senkirkine) in *Tussilago farfara* L. plants cultivated *in vitro*

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S u m m a r y

*Tussilago farfara* L. (family *Asteraceae*) is a valuable medicinal plant that has been used as a cough suppressant and as an antibacterial and anti-inflammatory drug. Mucopolysac-

charides, flavonoids, sterols, phenolic acids and pyrrolizidine alkaloids (PAs) are the main active compounds of coltsfoot. Due to hepatotoxic properties of some pyrrolizidine alkaloids, raw materials that contain PAs should be monitored and determined. The aim of present work was to establish nodal cultures of *Tussilago farfara* and to determine the content of senecionine and senkirkine in plants propagated in *in vitro* conditions. Eleven clones of coltsfoot derived from Polish natural populations were established. Rhizome buds were used as explants for the initiation of *in vitro* cultures on MS (Murashige and Skooge) medium. Every six weeks the shots and leaves were collected and dried. The HPLC method was applied for the identification and determination of senecionine and senkirkine. Content of pyrrolizidine alkaloids varied significantly depending on origin (population). An average sum of alkaloids (senecionine and senkirkine) ranged from 1.23 to 10.47 mg/100g d.w. that corresponds to 0.0013–0.011%, respectively.

***Key words:*** *Tussilago farfara L., nodal cultures, pyrrolizidine alkaloids*

## INTRODUCTION

Coltsfoot (*Tussilago farfara* L.) is a perennial plant, from *Asteraceae* family widespread in Eurasia. It is a valuable medicinal plant that has been used in traditional and contemporary medicine as a cough suppressant in upper respiratory tract disorders, asthma, bronchitis and as an antibacterial and anti-inflammatory drug. Leaves are herbal raw material. The coltsfoot's pharmacological properties are: antibacterial [1], anti-inflammatory [2, 3], antioxidant [3] and also some neuroprotective activities [4, 5]. Coltsfoot raw material contains: mucopolysaccharides, pectin, inulin, terpens: tussilagon, α- and β-amyrin, arnidol, faradiol [6], sterols: taraksasterol, β-sitosterol, esculetine, flavonoids (kaempferol, quercetin and its glycosides, rutin, hiperoside), phenolic acids (ferulic, caffeic, chlorogenic, galic and p-hydroxybenzoic acids), fatty acids (stearic and palmitic) and also bisabolen epoxides [2]. Coltsfoot also synthesizes pyrrolizidine alkaloids: senkirkine [7], senecionine [8], tussilagine and their isomers [9]. Senkirkine and senecionine are undesirable due to their toxic properties. Pyrrolizidine alkaloids that consist of an unsaturated necine base are toxic for humans and others mammals, whereas those with saturated necine moiety are non-toxic. During detoxification of PAs (metabolized by cytochrome P450 enzymes in liver), necine base is oxidized to pyrrolic esters (dehydropyrrolizidine derivates) that form adducts with proteins and nucleic acids resulting in cell toxicity or tumorigenecity [10-12]. Pyrrols react with endothelial cells of hepatic veins leading to hepatic Veno-Oclusive Disease (VOD) and liver failure [13]. PAs have been also detected as a contamination derived from animals food in milk, meat, eggs or honey [12]. The problem of PAs toxicity led to the legal restrictions in the sale of herbal products. The health authorities decided to limit the allowed dose of PAs to 10 $\mu$g per day in herbal products [14]. The legislation of some countries is more restrictive, for instance in Austria, registration of any plant products containing senecionine or senkirkine is not allowed.

The aim of present work was to establish nodal cultures of *Tussilago farfara* and determine the content of senecionine and senkirkine in plants propagated in *in vitro* conditions.

## MATERIAL AND METHODS

*Tussilago farfara* plants from eleven natural Polish populations were collected in order to establish nodal cultures. The plants originated from scattered localities in Western and South-Western part of Poland. Plant material was collected in October of 2007 and 2008. Rhizome buds were used as the explants due to short time of seed germination ability. The buds were sterilized as follows: 70% ethanol with a drop of Tween (15–30 s), commercial bleach (5% of active sodium hypochloride; from 10 to 20 min.) rinsed in sterile, distilled water four times. Nodal cultures were induced on MS medium [15] supplemented with sucrose (3%), with no addition of growth regulators. First shots were obtained within 2–4 weeks. The subsequent procedure was applied in accordance with the method described by Wawrosch *et al.* [16]. Cultures were maintained at standard conditions (temperature of 23±1°C, 16/8 h light/dark photoperiod, illumination of 40–50 mmol $m^{-2}s^{-1}$ photosynthetic photon flux intensity provided by cool white fluorescent light) in culture jars (250 ml capacity), 6 explants per each jar. The culture cycle lasted for nearly 6 weeks. After this period, the shots were cut into one-nodal fragments and transferred to fresh medium. Root induction was followed on the same medium within two weeks. Shots were collected for phytochemical analyzes every 6 weeks. Raw material was weighted and dried at room temperature.

Determination of pyrrolizidine alkaloids (senkirkine and senecionine) in plant material obtained from *in vitro* cultures was performed using HPLC method. Dried and powdered sample (5.0 g) extracted with 50% (*v/v*) methanol and water-methanol extracts were obtained. An assay was performed using HPLC-DAD Agilent 1100. Chromatographic separation was achieved on Hypersil BDS C8 250 × 4.6 mm, 5 $\mu$m (Thermo) column, run with A mobile phase containing 5 $\mu$M sodium hexanesulphonate in 1% phosphoric acid, and B phase – acetonitrile. Gradient eluent was set as follows: 0–7 min. 20%B, 7–25 min. 40% B, 25–28 min. 60% B, 28–33 min. 80% B, 33–35 min. 80% B, 35–40 min. 20% B. Flow rate: 0.8 ml/min.; temperature: 40ºC. The UV detection wavelength was set at 220 nm. Identification and quantification of pyrrolizidine alkaloids was based on the retention time and comparison of UV spectra with authentic standards of senecionine and senkirkine.

All experiments were conducted in triplicate and values are expressed as means ±SD.

## RESULTS AND DISCUSSION

Content of pyrrolizidine alkaloids varied significantly depending on the origin of clone line (population). An average sum of alkaloids (senecionine and senkirkine) ranged from 1.23 to 10.47 mg/100g of d.w. (fig. 1) that corresponds to 0.0013%–0.011%, respectively. Senecionine was detected at a level from 0.11 to 0.85 mg/100 g of d.w. (Fig. 2). Senkirkine was produced at much higher level – from 1.07 mg/100 g of d.w. to 9.18 mg/100 g of d.w. (fig. 3). It was calculated as equivalent of 10.72 ppm (locality BB) and 96.23 ppm (locality KK) of senkirkine, respectively.

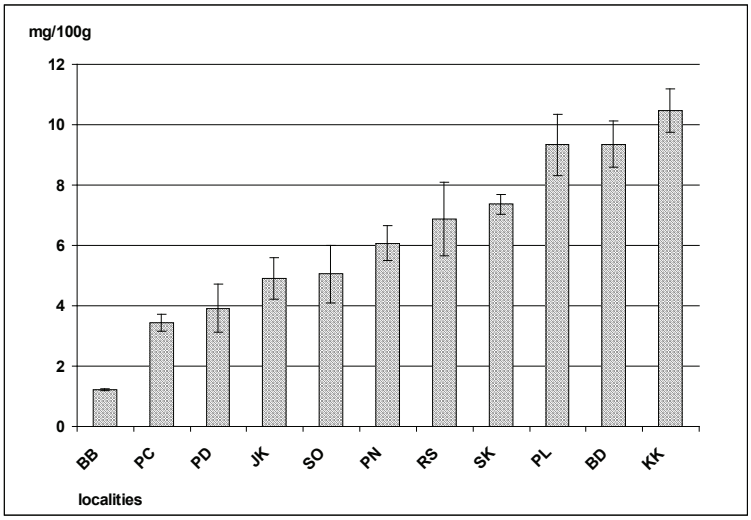


Figure 1.

Content of PAs in coltsfoot plants obtained from *in vitro* cultures. Values are expressed as mean ±SD of triplicate analysis. Localities: PD-Poznań-Dębiec, JK-Jóźwin, KK-Kamienica, SK-Szyszłowo, PC- Puńców, BB-Brenna-Bukowa, SO-Słomowo, PN- Poznań-Naramowice, BD- Borek-Deszczno, RS-Resko, PL- Plewiska

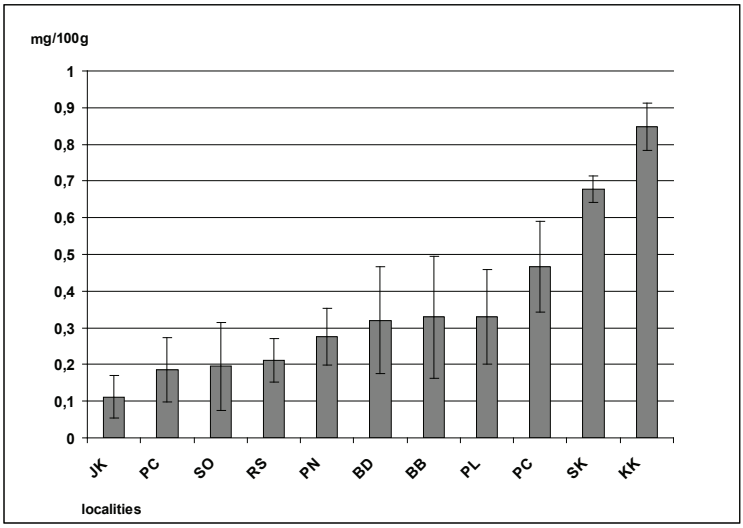


Figure 2.

Content of senecionine in *Tussilago farfara* plants. Values are expressed as means ±SD of triplicate analysis. Localities: PD-Poznań-Dębiec, JK-Jóźwin, KK-Kamienica, SK-Szyszłowo, PC-Puńców, BB-Brenna-Bukowa, SO-Słomowo, PN-Poznań-Naramowice, BD-Borek-Deszczno, RS-Resko, PL-Plewiska

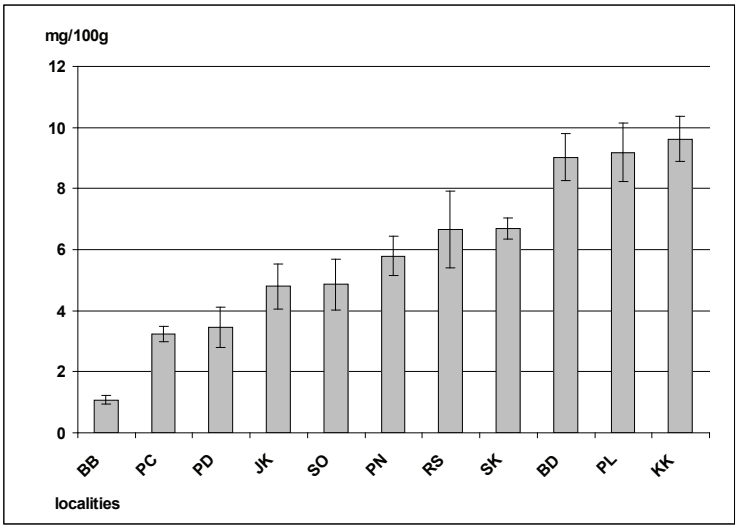


Figure 3.

Content of senkirkine in *Tussilago farfara* plants. Values are expressed as means ±SD of triplicate analysis. Localities: PD-Poznań-Dębiec, JK-Jóźwin, KK-Kamienica, SK-Szyszłowo, PC-Puńców, BB-Brenna-Bukowa, SO-Słomowo, PN-Poznań-Naramowice, BD-Borek-Deszczno, RS-Resko, PL-Plewiska

In general, PAs content is variable and depends on genotype, phase of growth and organ deposition. There is a very limited numbers of reports concerning PAs content in material derived from *in vitro* cultures. Wawrosch *et al.* [16] obtained nodal cultures from seeds and found free of alkaloids clone (Wien) that was patented. Micropropagated plants acclimatized and transferred to field conditions produced senkirkine at a level from 0.5 to 46.6 ppm. They also reported a very high concentration of senkirkine (up to 200 ppm and higher) in some clones. Senkirkine is the main pyrrolizidine alkaloid produced by *Tussilago farfara* plants. Senecionine is not always detected in raw material, usually its level is very low. According to Röder [17] coltsfoot raw material contains from 0.1 to 150 ppm of senkirkine. Mroczek *et al.* [18] detected 0.45 ppm of senkirkine (using HPLC method) in leaves and flowers and 92.8 ppm in rhizomes of coltsfoot. The amount of deposited senkirkine in raw material depends on the origin, part of plant and harvest time. Buchwald and Adamczak [19,20] reported that leaves of coltsfoot harvested in summer (June and July) contained from 0.5 $\mu$g to 136 $\mu$g PAs in 100 g d.w.

## CONCLUSION

Obtained results fall within the ranges reported by other authors, although content of PAs in *in vitro* material is higher than obtained from *in vivo* conditions. Usually, the material from *in vitro* cultures contain much lower level of alkaloids

compared to the intact plants. Probably some factors of *in vitro* conditions (temperature, light, nutrients etc.) or completely different life cycle are favorable for PAs biosynthesis. The subsequent studies are needed to confirm or to exclude this assumption.

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## ZAWARTOŚĆ ALKALOIDÓW PIROLIZYDYNOWYCH (SENECJONINY I SENKIRKINY) W ROŚLINACH PODBIAŁU POSPOLITEGO HODOWANEGO W KULTURACH *IN VITRO*

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S t r e s z c z e n i e

Liść podbiału pospolitego (*Tussilgo farfara* L.) jest cenionym surowcem zielarskim, stosowanym w stanach zapalnych i nieżytach górnych dróg oddechowych oraz jako środek przeciwkaszlowy i przeciwbakteryjny. Za główne substancje aktywne surowca (liście) uważa się przede wszystkim mukopolisacharydy, ponadto podbiał zawiera także flawonoidy, sterole, kwasy fenolowe oraz hepatotoksyczne alkaloidy pirolizydynowe, jak senkirkinę i senecjoninę. Celem pracy było założenie kultur węzłowych podbiału pospolitego oraz określenie zawartości senecjoniny i senkirkiny w namnażanych roślinach. W rezultacie otrzymano 11 klonów podbiału pochodzących z różnych populacji z terenu Polski. Do inicjacji kultur węzłowych użyto pączków bocznych (z kłączy) na pożywce wg Murashige i Skooga (1962) bez dodatku regulatorów wzrostu. Co sześć tygodni zbierano ulistnione pędy otrzymanych roślin, które następnie ważono i suszono w temperaturze pokojowej. Identyfikacji tożsamości i zawartości alkaloidów pirolizydynowych dokonano przy użyciu metody HPLC. Zawartość senkirkiny i senecjoniny wahała się w zależności od populacji. Średnia zawartość sumy alkaloidów (senkirkiny i senecjoniny) wynosiła od 1,23 do 10,47 mg na 100 g suchej masy, co odpowiada zakresowi od 0,0013% do 0,011%.

***Słowa kluczowe:*** *Tussilago farfara L., kultury węzłowe in vitro, alkaloidy pirolizydynowe*