P 2: The application of multi-shoots cultures in micropropagation of willow herb (*Chamaenerion angustifolium* (L.) Scop.)

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Abstract

Willow herb (*Chamaenerion angustifolium* (L.) Scop. syn. *Epilobium angustifolium* L.) from Onagraceae family is a valuable medicinal plant that has been used in the treatment of urogenital disorders including BPH (Benign Prostatic Hypertrophy). The raw material is a rich source of polyphenols as well as steroids, triterpenoids and fatty acids. The extracts show pharmacological activities: anti-androgen, anti-proliferative, anti-inflammatory, antioxidant, antibacterial and analgesic properties. Due to frequent interspecific hybridization, plants collected in the wild display a diverse and variable content of active compounds. This poses a challenge in obtaining high quality and homogenous raw material. Application of the *in vitro* cultures and micropropagation techniques may offer a solution for alternative methods of cultivation. This work presents preliminary results of the implementation of *Ch. angustifolium in vitro* cultures to obtain raw material for the first time. Sterile seedlings were donors of explants, which were used for induction of multi-shoots culture according to a modified Turker's protocol. Six different genotypes (lines) originating from root explants were chosen for clonal propagation. Efficiency of the elaborated method was 16 – 20 shoots per explants. Finally, over 3000 acclimatized plants were obtained and used for field crops.

Keywords: willow herb, *in vitro* culture, micropropagation, multi-shoots cultures.

Introduction

Willow herb (*Chamaenerion angustifolium* (L.) Scop. syn. *Epilobium angustifolium* L.) (Onagraceae family) can be found in Europe, Western Asia and North America. According to traditional folk medicine, its herb and roots were used in the treatment of gastrointestinal disorders, skin diseases and also of prostate, kidney and urinary tract disorders (VOGL et al., 2013). The raw material (*Epilobii herba*) is a rich source of polyphenols (flavonoids, phenolic acids, and tannins), steroids, triterpenoids and fatty acids (GRANICA et al., 2014). Oenothein B (macrocyclic ellagotanoid) is a major constituent of plant material (2 % - 14 %) and has been regarded as one of main active compounds. Anti-proliferative and antioxidant activity of the *Epilobium* extracts has been revealed (VITALONE et al., 2001, 2003; KISS et al., 2006). The studies on human prostate cancer cell lines (LNCP) have confirmed the anti-proliferative and anti-cancer effect of oenothein B (STOLARCZYK et al., 2013). The studies on willow herb extracts have induced growing interest in therapeutic potential of the *Epilobium* plants and their application in the treatment or prevention of BPH and other diseases. The great interest resulted in increasing demand on the raw material for the pharmaceutical and food industries. Nowadays, the crop plantations provide the majority of the raw material used for the medicinal or food products and only minority of them originate from the wild. It allows for obtaining large batches of high quality material and meeting the strict requirements for the medicinal products released on market. In case of *Ch. angustifolium* the raw material has been sourced from the wild. Due to frequent interspecific hybridization, the wild plants display a diverse and variable content of active compounds. Application of the *in vitro* cultures and micropropagation of selected genotypes offers an alternative way for traditional field cultivation. This work presents the preliminary results, where *in vitro* cultures and micropropagation technique were used for the first time in order to obtain willow herb raw material for the production of a dietary supplement used in the BPH prevention.

Materials and Methods

Ch. angustifolium seeds from the collection of Garden of Medicinal Plants of Institute of Natural Fibres and Medicinal Plants (INF&MP) were used for induction of *in vitro* cultures. Seeds were sterilized with use of 70 % ethanol and ACE® solution (2:1). Sterile seedlings were cuts for explants (roots, leaves, stem's segments and shoot tips) and placed on modified induction medium according to Turker (TURKER et al., 2008). Induction medium - MS medium (MURASHIGE and SKOOG 1962) supplemented with BAP (0.1 mg/L), IAA (0.5 mg/L) with vitamin C (0.1 g/L) and hydrolysate casein (0.5 g/L). After four weeks the obtained multiple-shoots were subcultured on fresh medium and the shoots were individually separated and transferred into vessels containing rooting medium: ½ MS with IAA (0.25 – 1.0 mg/L) with vitamin C (0.1 g/L). The number of shoots per explants was calculated from each ten multi-shoots individually separating shoots in three subsequent passages. Each seedling was marked by a number and multi-shoots and shoots derived from individual seedlings were represented by separated lines (genotypes) .The percentage of rooted plants was recorded after four weeks for 100 plants. All cultures were incubated in temperature 25 °C under the 16/8 h photoperiod (cool – white fluorescent lights 25 – 30 µmol m/l2s). The rooted plants were transferred into soil substrate and perlite (Kekkila Paperpot) and acclimatized in closed tunnels in the greenhouse conditions for two weeks. The plants were hardening in the open tunnels in temperature 16 °C for two weeks and for another two weeks in field conditions. All experiments were repeated three times.

Results

The best shoot regeneration was obtained from stem fragments (96 %) and root explants (60 %), which formed multi-shoots (table no 1). The rest of explants (leaves and shoot tips) only occasionally regenerated shoots, with a higher frequency of roots and callus formation. Browning of tissues, especially stem segments and leaves, leading to necrosis of explants was observed on induction medium in the subsequent passages.

Tab. 1 Shoot regeneration from explants: roots, stem's fragments, shoot tips and leaves on induction medium.

Explants	No of explants	**% explants forming shoots**	**% survived explants**
Roots	55	60 %	100 %
Stem's fragments	24	100 %	96 %
Leaves	123	10.7 %	83.7 %
Shoot's tips	15	25 %	80 %

Finally six different genotypes (lines) originated from root's explants were subcultured for over 6 months (passages numbers from 2 to 23). The numbers of shoots and multi-shoots (calculated for all lines) changed between single subcultures. The efficiency of shoot production was variable from passage to passage and depended on line. Average numbers of shoots calculated for explants varied between genotypes and oscillated between 16.1 and 20.4 (Figure 1).

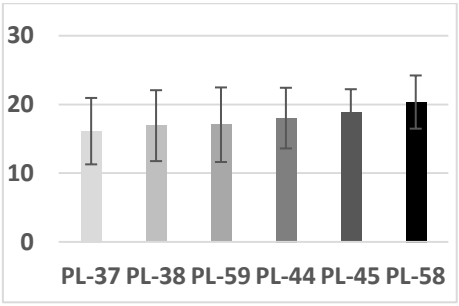

Fig. 1 Average number of shoots per explant of individual lines calculated in subsequent passages: 21, 22, 23. Error bars express SD.

The shoots were rooted on medium containing three different concentrations of IAA (0.25 mg/L; 0.5 mg/L and 1.0 mg/L). The optimal concentration of IAA was 0.5 mg/L and 98 % (±1.01) of healthy and rooted plants were recorded. The whole cycle lasted from 10 to 12 weeks from seedlings to acclimatized plants. The acclimatization resulted in high survival rate (98 %) of rooted and hardened plantlets. Finally, over 3 000 acclimatized plants were obtained and used for field crops.

The modified Turker's protocol is an efficient and rapid method, which allows for obtaining of a high number of shoots (16 - 20) per explant. Genotype dependent response of the explants was observed during regeneration. The major problem was browning of tissues and necrosis of the explants, what limited the number and quality of the regenerated shoots. The use of *Ch. angustifolium in vitro* cultures can contribute to the introduction of this valuable herb species for field crops and increase the availability of the raw material for food and the pharmaceutical industries.

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