



ENVIRONMENTAL AND ECOLOGICAL ASPECTS OF CULTIVATION OF SELECTED ENERGY AND HERBAL PLANTS PROPAGATED BY *IN VITRO* CULTURE

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Summary

There has been a growing interest over the last years in techniques using alternative and pharmaceutical plants due to their wide potential applications. These species are characterised by valuable and multidirectional usable features, due to which their practical application is superior to their physiognomic features. These plants may provide a raw material for production of medicines, cosmetics or may be used for animal feeds. Moreover they may be used as ornamental, melliferous and energy plants. Owing to their generally low soil requirements they are used in the reclamation of degraded land. Among the species characterized by multidirectional use are *Silphium perfoliatum*, *Helichrysum arenarium* and *Stevia rebaudiana*. These plants are characterized by a high content of active compounds and a number of pro-health characteristics. Because raw materials for production of medicines and cosmetics should be standardized, cultures maintained under controlled conditions are a good source of seedlings.

Key words: *Silphium perfoliatum*, *Helichrysum arenarium*, *Stevia rebaudiana*, micropropagation, growth regulation,

INTRODUCTION

Plants crucially affect the state of the natural environment in which we live, they are also vital for protection of human health. Fundamental importance of plants for the condition of the natural environment results from many reasons

among other including the ability for phytoremediation or phytostabilisation of soils. Numerous plant species find applications in protection of human health, as various medicines or in a form of active substances extracted from them. Micro-propagation is the method allowing to obtain a big number of seedlings genotypically and phenotypically equalized and free of pathogens and pests. Owing to cultures maintained *in vitro* it is possible to recreate and restore threatened species to the natural environment, as well as propagate these which occur only endemically, remain under partial or total protection. *In vitro* techniques became an indispensable element of programmes aiming at creating new, or donor materials for breeders, but also for improvement of crops or ornamental plants species, as well as species in pharmaceutical or cosmetic industry. Dissemination results of research on traditional field crops contributed to a fast progress in propagation of valuable genotypes in production of homozygous lines (Gürel *et al.* 2000, Kuykendall *et al.* 2003, Mezei *et al.* 2006). Owing to the application of *in vitro* cultures both shortening and improvement of traditional breeding cycle are possible. However, the process requires development of efficient and repeatable plant regeneration procedures in controlled conditions, which will make possible their implementation in agricultural practice. The material initiating plant cell and tissue cultures may include meristem cells, petioles and leaf blades, cotyledons, hypocotyls, anthers, germs and protoplasts. Development and morphogenesis of explantants depend on numerous cooperating factors connected both with the nature of plant material and *in vitro* conditions.

Silphium perfoliatum

In the natural environment *Silphium perfoliatum* L. occurs in the central and eastern part of the United States and in the south of Canada (Stanford 1990, Rutkowski 2011, Wróbel *et al.* 2013). The species may be used for reclamation of degraded areas, particularly for phytoremediation, for removal or detoxication of pollution from the environment (Klimont 2007, Majtkowski 2010). It may be also treated as a potential species for cadmium phytostabilisation in soil (Zhang *et al.* 2010).

Due to high concentration of carbohydrates, minerals and crude protein, rich in exogenous amino acids it may be also useful for farm animal feeding (Lehmkuhler *et al.* 2007, Piłat *et al.* 2007, Țiței *et al.* 2013). On the other hand, inulin (polysaccharide) isolated from rhizomes and roots of *Silphium perfoliatum* L. functioning as prebiotic may be used in dietetics, veterinary medicine and cosmetics (Kowalski and Wierciński 2004).

Medicinal properties of *Silphium perfoliatum* L. are determined by biologically active secondary metabolites. Therefore, extracts from this species tissues reveal pain-killing, anti-inflammatory, diaphoretic, restorative, antibacterial, antifungal and expectorant properties, as well as decrease cholesterol level. So

far, the following compounds have been discovered in various organs of *Silphium perfoliatum* L.: terpens, essential oils, triptenoid saponins (oleanosides), phenolic acids, tannin-tan compounds, carotenoids and flavonoids (Kowalski 2002; Kowalski and Wolski 2003; Kowalski and Kędzia 2007, Jemiołkowska and Kowalski 2012).

Both numerous usable features of *Silphium perfoliatum* L. and climatic conditions approximate to these in which it naturally occurs may favour this species cultivation in Poland. However, the sources of seeding material available in Poland do not satisfy growing demand of breeders. The technology which enables obtaining vegetative propagation material is plant micropropagation in the *in vitro* cultures. The research on obtaining micro seedlings used as initial material the seeds of *Silphium perfoliatum* L., previously subjected to sterilization process. The most efficient method proved to be germ isolation and treatment with 70% ethyl alcohol for 30 seconds and subsequently by 30% ACE (commercial bleach with sodium hypochloride as one of its components (4.85%) and other compounds containing <5% of chlorine) with a supplement of 2-3 drops of Tween 20 for 7 minutes, followed by thrice rinsing in bi-distilled water (Figas *et al.* 2010, Tomaszewska-Sowa and Figas 2011). Explantants used in the experiment comprised fragments of cotyledons, lead petioles and top parts of shoots. The highest percentage of explantants with lateral shoots (84.2-90.3%) and callus tissue (41.7-92.8%) was obtained on top explantants of seedlings placed on the MS medium (Murashige and Skoog 1962) with an addition of 5mg·dm⁻³BAP (benzylaminopurine) and 1mg·dm⁻³ NAA (naphthalene-1-acetic acid) (Figs *et al.* 2010, Tomaszewska-Sowa and Figas (2011)). As has been demonstrated by numerous experiments, induction of rhizogenesis was achieved on MS medium with BA (benzyladenine) and IBA (indole-3-butyric acid) (SHI Xiang-yuan *et al.* 2011) or without growth regulators (Figas *et al.* 2010, Tomaszewska-Sowa and Figas 2011).

***Helichrysum arenarium* (L.) Moench**

Helichrysum arenarium (L.) Moench is a trailing perennial from the *Asteraceae* family. In Poland the plant is under partial protection due to valuable medicinal properties and applications in the pharmaceutical industry. In medicine *Helichrysum* inflorescence or flowering heads are used. Medicinal properties of this plant are determined by active substances – flavonoids present in the raw material, which reveal holagogue, diastolic and antibiotic effect. Therefore, *Helichrysum arenarium* (L.) Moench found applications in the treatment of alimentary tract and liver diseases (Bacler 2009).

In case of herbal plants *in vitro* cultures provide a chance for obtaining a valuable and homogenous raw material. The investigations on micropropagation of *Helichrysum arenarium* made use of the plant material originating from

natural sites. The initial explantants were top buds covered by two leaves. An efficient method of their sterilization relied on disinfection for 1-2 seconds in 70% ethanol solution and then for 10 minutes in 9% calcium hypochloride solution with Tween 20 addition. Sterilized buds were placed on MS medium enriched with $30\text{mg}\cdot\text{dm}^{-3}$ of thiamin, $10\text{mg}\cdot\text{dm}^{-3}$ of nicotinic acid, $40\text{mg}\cdot\text{dm}^{-3}$ of glycine and $1\text{mg}\cdot\text{dm}^{-3}$ KIN. Single shoots obtained at this stage were transferred onto MS media with various combinations of growth regulators. The highest propagation coefficient was obtained on MS media with a supplement of $4\text{mg}\cdot\text{dm}^{-3}$ KIN, $0.3\text{mg}\cdot\text{dm}^{-3}$ BAP and $0.1\text{mg}\cdot\text{dm}^{-3}$ NAA, respectively 16.10 and 23.35 of shoots per explantant (Sawilska, Figs 2006).

In the investigations of Pawelczak and Bryksa-Godzisz (2008) the initial material were seeds disinfected in the 10% solution of commercial Domestos (cleaning and disinfectant preparation containing sodium hypochloride in concentrations from 3.68 to 5.62%), which were put on LS medium (Linsmaier and Skoog 1965) containing a half of the concentration of the elements in relation to the original medium. From the seedling obtained in this way top growth explantants were isolated and transferred onto the MS and MS/B5 propagation media (modified medium containing inorganic macro – and microelements from MS medium and vitamins from B5 medium (Gamborg *et al.* 1968), supplemented with 6-benzyladenine (BA) in concentrations of 0.1 and 1.0 $\text{mg}\cdot\text{dm}^{-3}$. The number of shoots obtained from one explantant was higher on media enriched with $1.0\text{mg}\cdot\text{dm}^{-3}$ BA (15.85 – 17.59 shoots/explantant) than on the other variants.

Stevia rebaudiana

Originating from South American countries *Stevia rebaudiana* Betoni is described as “sweet plant” (Lemus-Mondaca *et al.* 2012), which is connected with its great sweetness even 300 times higher than sucrose. In contrast to the latter, stevia extracts do not cause rapid changes of blood glucose level, moreover they reveal antibacterial, antifungal, antiinflammatory and blood vessels widening properties (Goettemoeller and Lucke 2010). Due to thermostability within a wide temperature range and pH they provide an excellent supplement to many food categories. Numerous advantages of steviol glycosides cause an apparently growing interest in this species among food and medical industries. This hypocaloric and dietetic product excellently fits into contemporary trends of promoting healthy and organic food. In conditions of traditional cultivation, obtaining a large number of plants is difficult due to low seed germination capacity and their low vigor. In this situation application of properly selected *in vitro* culturing method seems the optimal solution (Jain *et al.* 2009, Alhady 2011, Jitendra *et al.* 2012).

The explantants used for initiating this plant species culturing were top fragments of shoots (Ibrahim *et al.* 2008, Das *et al.* 2011), nodal sections (Ahmed

et al. 2007, Alhady 2011, Das *et al.* 2011, Jitendra *et al.* 2012, Thiyagarajan and Ventkatachalan 2012) and isolated buds (Das *et al.* 2011). The highest number of explantants which started growing was observed on MS medium in the presence of $0.5\text{mg} \cdot \text{dm}^{-3}$ BAP and $0.5\text{mg} \cdot \text{dm}^{-3}$ KIN (Alhady 2011). On the other hand, the efficiency of lateral buds development was intensified by BAP presence in the medium (Thiyagarajan and Ventkatachalan 2012). As was demonstrated by experiments, the highest average number of buds from a single explantant was obtained under the influence of applied quite high BAP concentrations ($1.0 - 2.0\text{mg} \cdot \text{dm}^{-3}$) (Ibrahim *et al.* 2008, Thiyagarajan and Ventkatachalan 2012) or under the influence of BAP and KIN complex ($0.5\text{mg} \cdot \text{dm}^{-3}$) (Ahmed *et al.* 2007, Alhady 2011). As has been reported by Jitendra *et al.* (2012) and Thiyagarajan and Ventkatachalan (2012) including IAA, NAA or IBA in the medium composition improved the effectiveness of the proliferation process, however the obtained shoots revealed short internodes. According to Das *et al.* (2011) presence of high concentrations of several different phytohormones from auxins and cytokinins group may have negatively affected shoot genesis processes. Shoot formation might have been inhibited in these cases by calluses forming on the explantants. Dwarfiness of regenerated stevia shoots was overcome through enrichment of the medium in KIN. In this respect, the most efficient proved concentrations of 2.0 (Das *et al.* 2011) and $1.0\text{mg} \cdot \text{dm}^{-3}$ (Ibrahim *et al.* 2003), as well as a combination of KIN with BAP at $0.5\text{mg} \cdot \text{dm}^{-3}$ BAP and $0.5\text{mg} \cdot \text{dm}^{-3}$ (Jitendra *et al.* 2012) and also 5.9cm at $0.5\text{mg} \cdot \text{dm}^{-3}$ BAP and $1.0\text{mg} \cdot \text{dm}^{-3}$ KIN (Alhady 2011).

Callus formation was observed during stevia micropropagation process, which allowed for obtaining shoots also through indirect organogenesis. Fragments of young leaves were used for induction of this tissues (Uddin *et al.* 2006, Janarthanam *et al.* 2009, Moktaduzzaman and Rahman 2009, Mathur and Shekhawat 2013, Gauchan *et al.* 2014), nodal sections (Uddin *et al.* 2006, Janarthanam *et al.* 2009) and intermodal shoot sections (Uddin *et al.* 2006). Among the analysed explantants, the internodal sections were characterized by the highest potential for callus formation. The growth regulator which to the highest degree induced callus development were $\text{mg} \cdot \text{dm}^{-3}$ 2,4-D (dichloroacetic acid) (Uddin *et al.* 2006). Similar effects were obtained at the application of a complex of phytohormones from auxins and cytokins group. $1.5\text{mg} \cdot \text{dm}^{-3}$ of NAA and $1.0\text{mg} \cdot \text{dm}^{-3}$ of BAP applied by Moktaduzzaman and Rahman (2009) and $2\text{mg} \cdot \text{dm}^{-3}$ of NAA and $2.0-3.0\text{mg} \cdot \text{dm}^{-3}$ of BAP which were the medium components in the research of Mathur and Shekhawat (2013) stimulated formation of callus tissue on the explantants. A high percentage of callus forming explantants were obtained also due to the application of $1.0-2.0\text{mg} \cdot \text{dm}^{-3}$ of 2,4-D and $1.0-1.5\text{mg} \cdot \text{dm}^{-3}$ of NAA (Gauchan *et al.* 2014), as well as $2.5\text{mg} \cdot \text{dm}^{-3}$ of 2,4-D and $0.5\text{mg} \cdot \text{dm}^{-3}$ of BAP (Janarthanam *et al.* 2009). KIN and IBA media enrichment caused very weak callus induction, below 50% (Gauchan *et al.* 2014). Improvement of *in vitro* regeneration techniques is necessary to make stevia production on a large scale

less complicated and available. The species improvement by means of genetic engineering is also important (Jagatheeswari and Ranganathan 2012, Modi *et al.* 2012).

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