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REVIEW ARTICLE

Application of *in vitro* stevia (*Stevia rebaudiana* Bertoni) cultures in obtaining steviol glycoside rich material

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Summary

Stevia is a plant attracting attention due to its capability to synthesize a group of chemical compounds with sweet taste, i.e. steviol glycosides. Steviol glycosides are successfully applied as a natural sweetener, and some of them have also therapeutic properties. This paper presents available information on the use of stevia plant tissue cultures with the focus on their potential application in food industry. Detailed analysis was done concerning the research employing *in vitro* culture techniques and the use of them in biosynthesis of secondary metabolites of high importance for the food industry. Both established achievements and most recent publications on stevia were used for assessment of practical applications of the aforementioned techniques and prospects for their development.

Key words: *steviol glycosides, in vitro cultures, secondary metabolites, micropropagation, Stevia rebaudiana, stevia, stevioside*

INTRODUCTION

Plant cell and tissue cultures are used in plant breeding. Thanks to their use it is possible to investigate the plant growth, development and metabolism. They allow for massive vegetative reproduction and/or propagation of plants that cannot be reproduced in natural conditions by micropropagation or somatic embryogenesis.

In vitro cultures also enable to obtain hybrid plants, even when crossbreeding the plants with morphological or physical barriers prohibiting pollination, and to obtain somatic hybrids. Anther and microspore cultures allow for production of haploid and doubled haploid plants i.e. organisms that are totally homozygotic. The *in vitro* techniques find applications in the processes of inducing mutations in plant cells and of genetic transformation and finally of obtaining plants free from viruses.

Stevia (*S. rebaudiana* Bertoni) is a perennial herbal plant native to tropical and subtropical regions of North and South Americas. In natural conditions *S. rebaudiana* grows in a form of a shrub and reaches even 1 m of height [1]. Stevia is a short-day plant and its flowering is induced when days become shorter. However, a long photoperiod stimulates leaf growth and steviol glycoside production. The plant can survive only mild winters of its native regions. Longer exposure to temperatures lower than –4°C makes it impossible for stevia to survive winters, therefore, in colder regions it is cultivated as an annual plant. A crucial characteristics of stevia that draws attention to the plant is its capacity to synthesize a group of chemical compounds with sweet taste the so called steviol glycosides. This group comprises eight identified compounds with stevioside and A and C rebaudioside being the most common. The remaining compounds i.e. B, D, F rebaudiosides, steviolbioside and dulcoside are present in much lower amounts [2, 3]. It is estimated that steviol glycosides at the concentration of 4% are even 300 times sweeter than saccharose at the same concentration [4]. Steviol glycosides are successfully used as a natural sweetener; some of the compounds produced in stevia also show therapeutic properties. Stevia is non-toxic, non-carcinogenic and non-mutagenic [5]. Additionally, the plant and its extracts are characterized with low glycemic index, thus, do not affect glycaemia. The plant shows blood pressure lowering properties, also antibacterial, antifungal and diuretic properties [3]. Currently, stevia is used both directly and after processing as a sweetener for tea, chocolate, jam, cookies, ice cream, juice and other soft drinks and yoghurt etc. [6]. The ease of applying stevia results from a few of its features, namely, the leaves do not require expensive processing and can be used either directly or dried and powdered; also extracts and powders are commercially available. Due to its intensity, only small amount is needed for sweetening thus the processing and transportation are facilitated. Steviol glycosides are not a substrate for fermentation, therefore, do not decompose easily. Moreover, they are stable up to 200°C, what implies that they can be used as a sweetener for cakes. An evident advantage of the plant is its well established long-term safety of use [7].

One of the most important ways of using *in vitro* techniques in breeding of stevia is its micropropagation. It is the fastest and most efficient method of obtaining a high number of plants in industrial scale, the plants homogenous in terms of composition and contents of steviol glycosides and free of diseases [1]. The usefulness of the method is further enhanced by the fact that stevia seeds are characterized with very low germination strength [8], and generative propagation results in plants of varied genotypes and phenotypic traits, what does not allow for obtaining homogenous population in terms of such important traits as the

content of steviol glycosides and chemical composition. Searching for solutions within traditional vegetative reproduction/propagation methods requires a lot of efforts and their efficiency is limited with the availability of the genetic material. The literature on the subject suggests that biotechnological methods are expected to bring improvement in cultivation [9, 10].

The effect of culture media on micropropagation processes

The effect of mineral components

Mineral nutrients are the basic elements of media used in plant *in vitro* cultures. Their concentration and ratio within the medium play a fundamental role in determining the rate and quality of the material growth, therefore when selecting medium composition both parameters must be specified [6].

Ibrahim et al. describe the effect of different concentrations of MS medium and its main components on the *in vitro* development of stevia. The best results were achieved when using MS medium and MS modified in the following way: NH_4NO_3 at a concentration of 1237.5 mg/l, KNO_3 950 mg/l, $MgSO_4$ 185 mg/l, $CaCl_2 \cdot 2H_2O$ 440 mg/l, KH_2PO_4 85 mg/l, enriched with microelements, saccharose at 30 g/l, glycine 2 mg/l, pyridoxine 0.5 mg/l, nicotinic acid 0.5 mg/l, thiamine 0.1 mg/l, and also solidified with agar with 1.5 mg/l Gelrite [6]. Doubled concentrations of mineral salts in MS medium has a positive effect on the plant growth but results in a drop in steviol glycoside levels by an order of magnitude [11].

The effect of the composition and concentration of microelements on the shoot induction and chlorophyll content was studied by Jain et al. [12]. The team used media with different concentrations of cobalt, iron, manganese, copper and potassium iodide and compared with the results obtained on standard MS medium. A positive effect on shoots induction were observed for the elevated concentrations of $MnSO_4$ (60.4 mg/l), $CoCl_2$ (0.26 mg/l) and KI (1.7 mg/l), while proliferation was enhanced with: $CoCl_2$ (0.26 mg/l), $CuSO_4$ (1.6 mg/l), $MnSO_4$ (75,.5 mg/l), $ZnSO_4$ (14.4 mg/l), and H_3BO_3 (24.7 mg/l). The growth in biomass production and chlorophyll content was observed for elevated levels of $Fe_2(SO_4)_3$ (80.0 mg/l), $CoCl_2$ (0.4 mg/l) and KI (1.7 mg/l) [12]. Other literature sources indicate that copper at 0.03 mg/l has a positive effect both on induction and proliferation of shoots and also on chlorophyll content and biomass [13].

The effect of carbon sources

Sugars provided for the culture in medium are a source of carbon and energy, what highly accelerates their growth and development, making it independent from photosynthesis. This process is hindered in *in vitro* conditions as gas

exchange is inhibited. Thus, the availability of carbon dioxide for the photosynthesis. Additionally, not all the cells grown *in vitro* are capable of photosynthesizing and the presence of carbohydrates is essential for them. On the other hand, the presence of sugars can enhance growth of microorganisms on the medium.

The effect of the sugar type and concentration on the development of stevia shoots and content of steviol glycosides in the biomass placed in a bioreactor was studied by Bondarev et al. [11]. In the medium containing fructose or glucose the development of the root system was more advanced, yet the leaf mass decreased as well as steviol glycoside levels, as compared with the saccharose-containing medium. For improvement the content of steviol glycoside in the tissues different concentrations were tested (1–5%), while the optimal saccharose concentration was 3%, with higher concentrations enhancing the development of the root system and biomass accumulation [11].

The effect of growth regulators

Plant growth and development regulators are a group of compounds regulating biochemical processes in plants. They are responsible for proper growth and cell division as well as plant response to changing environmental conditions e.g. reaction to stress. A plant reaction to these compounds depends on the concentration and can be observed even at small amounts of used preparations.

In *in vitro* cultures growth regulators are applied in order to induce certain behavior in the cultures e.g. development of adventitious buds, callus development or rooting. Selection of proper concentration of these compounds is crucial for quality and quantity of the plant material. The literature on the subject presents numerous experiments on optimizing the concentrations of growth regulators on stevia micropropagation.

The studies on differences in tissue development of stevia *in vitro* cultures under the effect of MS medium supplemented with 2.0 mg/l BA (6-benzyl amino purine) recorded the maximum number of shoots of 43.9 shoots/explant, but these shoots were very thin, containing many lateral shoots, but with low survival percentage during acclimatization [9]. The effect of various concentrations of BA, IAA (indole-3-acetic acid) and IBA (indole-3-butyric acid) on MS medium carried out by Sivaram and Mukundan (2003) showed that the most efficient combination for shoot regeneration is BA at 2 mg/l combined with IAA at 1 mg/l, what results in multiplication rate at 11, 10 and 8 for explants of apical bud, adventitious buds and leaves [1]. Lower efficiency is observed in case of an IBA supplemented medium, which – when diluted doubly – worked well for rooting the shoots, where 100% root regeneration was achieved. Such a composition of the medium had also a positive effect on shoot elongation. Other combinations of the regulators i.e. BA with NAA (1-naphthaleneacetic acid), KIN (kinetin) with auxins turned out to be

less efficient. The authors estimated that from a single explant after 6 months and including the plant adaptation process to *ex vitro* conditions about 27 000 plants can be produced.

The literature on stevia describes several other experiments with stevia micropropagation. Bondarev et al. [11] observed 1.5 more shoots produced from explants, although, rhizogenesis inhibition under the influence of 0.1 mg/l BA at the same time, as compared with the standard medium. On the other hand, gibberellic acid (GA_3) caused elongation of shoots and roots [14]. Jain et al. [13] observed optimal shoot induction with the use of BA at 0.5 mg/l and IAA at 0,5 mg/l. In case of shoot proliferation the best results were achieved when using a combination of BA (0.8 mg/l) and KIN (0.4 mg/l). The initial explants in those studies were leaves and adventitious buds [13]. Another source reports a different optimal combination of growth regulators in MS medium at MS 0.3 mg/l KIN for shoot initiation, and 2 mg/l IBA for root induction [15].

The studies reported by Anbazhagan et al. [16] were done also for MS medium supplemented with BA, KIN and IAA separately and in combinations. The best results were observed for the MS medium with an addition of BA at 1 mg/l and IAA at 0.5 mg/l, what is consistent with earlier publications. The explant of the highest propagation rate was also an apical bud. However, the researchers indicate that for root regeneration N6 (Nitsch) medium with IAA at 1 mg/l is the most effective [16].

Other cytokine combinations, used by Yadav et al. [17] for induction of adventitious buds, comprised BA with KIN or NAA. The best results were observed when using MS medium with BA at 0 5 mg/l and KIN at 0.5 mg/l. The highest shoot elongation and development of the highest number of leaves occurred under the effect of GA_3 at 0.5 mg/l added to MS medium. Rhizogenesis proceeded in the most effective (87.8%) and fastest way with the use of 2 mg/l IBA, while the tests also included NAA and IAA. On the contrary, there was no root growth on MS medium without any growth regulators [17].

Thiyagarajan et al. [13] wrote on an explant culture of adventitious buds and stem nodes obtained earlier from another *in vitro* culture on MS medium with different concentrations of BA and KIN, in the other case supplemented with different concentrations of IAA, IBA and NAA. The highest propagation rate was observed for the material grown on a medium with BA at 1 mg/l, where the rate reached 15.69 buds per explant, with the frequency of occurrence at 94.5%. The experiment aimed at a large scale production of nodal explants and adventitious buds, which were then transferred to a MS medium with 1 mg/l BA. After three propagation cycles, 123 buds were obtained from each explant, which were later transferred to ½ MS medium with various concentrations of NAA, IAA and IBA auxines, what was successful. The highest share of rooted plants was achieved on ½ MS medium with NAA at 0.4 mg/l [3].

An interesting solution involved chlorocholine chloride (CCC) in combination with IBA to obtain optimal *in vitro* development of stevia, which was proposed by

Dey et al. [18]. The studies showed that the cultures grown on a medium supplemented with CCC and IBA developed better and showed more desired traits than those grown on media with CCC or only with IBA. The medium containing 3 mg/l CCC with 3 mg/l IBA proved to be the most effective both for increase of bud numbers, of biomass and chlorophyll content, for improvement of survival rate and finally for the content of stevioside [18].

To sum up, the literature data suggest most often a composition of exogenic plant growth regulators for bud/shoot induction as a MS medium with BA cytokines at 1 mg/l or BA at 1 mg/l with addition of 0.5 mg/l IAA. The explant providing the highest propagation rate is the apical bud. Rooting and elongation growth was the most efficient on a MS medium or its twofold dilution with the addition of the auxines, i.e. IBA at 2 mg/l or NAA at 0.4 mg/l.

The effect of use of Stevia callus in vitro cultures

Callus tissues under *in vitro* cultures, because of its totipotency leading to tissue differentiation, is used in indirect organogenesis or forming somatic embryos. Callus cultures are applied commonly for obtaining plant metabolites.

According to the literature concerning stevia, callus induction proceeded most efficiently on complete MS medium with 0.1 mg/l of 2,4-dichlorofenoxyacetic acid (2,4-D) [15] or with BA at 2–3 mg/l and NAA at 2 mg/l [19]. The development of buds from callus tissues was supported by supplementation with BA and 2,4-D to the MS medium MS [15]. Callus development was not observed on the pure medium with no supplementation with exogenic growth regulators, what indicates that the process is complex and requires the supplementation of the medium with the growth regulators [19].

The literature also provides information on regeneration of the plants by somatic embryogenesis where embryogenic callus was formed both from leaf explant and from floret explants [20].

The effect of Stevia in vitro suspension cultures

The optimal growth of suspension cultures of stevia was observed for the media with addition of 0.06 mg/l 2,4-D, 0.6 mg/l BA and 0.01 mg/l ascorbic acid, for which combination the specific growth rate was 2.6 1/day [19]. The authors indicate that the following modifications of mineral salt concentrations to the standard MS medium are optimal for the development of stevia suspension cultures: NH_4NO_3 at 2 mg/l, 5.7 mg/l KNO_3, 0.5 mg/l $MgSO_4$ and 10.2 mg/l KH_2PO_4. Better effect of elevated concentrations of salts on stevia is also confirmed by other authors [21].

The effect of acclimatization of plants from in vitro to ex vitro

Transfer of the plants from sterile *in vitro* conditions to external environment and their acclimatization are processes linked with numerous stress inducing factors. The plant must develop a functional root system, efficient transpiration mechanism, protective layers and start photosynthesis.

Substrates used in acclimatization of stevia are mixtures of soil and sand [19, 22], vermiculite [16], vermicompost [22], or coir peat [16]. Comparative analyses indicated for use of mixtures of sand, soil and vermicompost in ratio 1:1:1, where the plant survival rates at 82% [16], and also for use of coir peat alone with the survival rate at 70% [16].

Influence of several factors on steviol glycosides production in Stevia *in vitro* cultures

Steviol glycosides are the compounds responsible for sweet taste of stevia with the stevioside and A and C rebaudiosides as most common. Each of the compounds amounts at 1% or more of leaf dry mass in stevia grown in a traditional way. The content of stevioside, a rebaudioside and C rebaudioside reaches 3300 μg/g, 1900 μg/g and 700 μg/g of dry mass, respectively [14]. The remaining steviol glycosides are present in much lower amounts. The data on the content of steviol glycoside in plant tissues from *in vitro* differ very much in the literature with some of contradictory data. Swanson et al. [23] did not document stevioside presence either in callus cultures or in buds. They suggested that only a fully developed plant can produce these compounds. Other authors showed the capacity of bud cultures to synthesize these compounds [14, 18]. In the literature on callus and suspension cultures some authors did not report on any presence of steviol glycosides [14], some suggested it in the callus cultures [22], even at levels twice as high as in stevia leaves [24].

The studies published in 2001 showed that the stevia leaves and stems grown *in vitro* are characterized with respectively 5 and 3 times lower content of steviol glycosides than the same organs in plants grown in the traditional way [14]. Callus cultures practically did not contain any glycosides, what is contradictory with the results obtained by Taware et al. [15], who showed high content of stevioside in the callus cultures [15].

The content of steviol glycosides in suspension cultures varies, depending on a source, between 15 μg/g [14] and even 380, 3 μg/g [19] and shows a characteristic dynamics related to the cell propagation cycle. The maximum values are observed during the phase of logarithmic growth or at its end, and then during a stationary phase their content drops [19]. This indicates for a positive correlation of growth and cell division in suspension cultures and synthesis of steviol glycosides.

The medium composition, e.g. the content of mineral salts, the type and concentration of sugars and growth regulators, all may have an effect on the content of the steviol glycoside levels in the obtained material [20]. Exogenic growth regulators almost always contribute to the drop in steviol glycoside levels [11]. This might partially explain such varied concentrations in different culture types. However, no correlation was found between the explant type and the content of the glycosides in its equivalent in *in vivo* [14]. Cell differentiation seems to have influence on cell production of steviol. According to Bondarev et al. the content of these compounds in morphogenic callus was 4–5 times higher, and in case of formed buds even 37 times higher than their content in suspension cultures [14]. The role of cell disaggregation is also suggested in the process of steviol glycoside synthesis [19].

Chemical structure of steviol glycosides is of high importance in their production as it determines their taste properties. The differences in the steviol glycoside, specifically in the sugar residues substituted to aglycone, occur due to the presence and activity of numerous glycosyltransferases [25]. This diversity of glycosides is reflected in their properties, especially in perceived taste and solubility in water. In general, sweet taste is perceived when a hydroxylic or a sugar group is present in the C-19 position and the differences in the substituted groups in these positions determine the compound properties [26]. For example, rebaudioside A has more sought for taste than stevioside, which is characterized with specific aftertaste, perceived by some people as unpleasant or bitter, besides it is less sweet than rebaudioside A [27]. It was observed that stevioside and rebaudioside A have their equivalents, i.e. dulcoside A and rebaudiozside C, respectively, where glucosyl group is substituted with a rhamnose [26]. The studies carried out so far on glycosides based on aglycones other than steviol (isolated from *Stevia paniculata*, *Stevia ovata* and *Rubus suavissimus*) show that steviol yields the sweetest glycosides and that the taste of the steviol glycosides depends on its structure [26]. Therefore, some attempts have been made to modify their chemical structure for improvement of the taste. Due to its natural origin (and food applications) enzymatic modification methods are preferred to the chemical ones. Biotransformation is an enzyme catalyzed process of transforming of chemical compounds. Controlled biotransformation has been applied in modification and production of economically valuable compounds. One of such biotransformation reactions can be modifications of glycoside groups of a compound, joining, disconnecting and transglycosylation of the groups. In general, biocatalysis is a useful tool, for example in synthesis of specific enantiomers, regioselective synthesis, synthesis of compounds of lower toxicity or activity. It is an alternative for chemical synthesis as it usually does not require aggressive reaction conditions and does not generate hazardous wastes. It results in processes more friendly both for humans and environment. Cycloglucotransferase (CGTase) reacts with starch to form cyclodextrins and also catalyses trans-α-1,4-glucosylation. Stevioside was subjected to action of CGTase in the presence of soluble starch and the obtained

glycosides were then analysed. Improvement of taste was achieved for most glucolysed compounds, although glucosylation in C-19 position affected negatively the taste. This dependence was later used in other reactions with the use of more specific enzymes so that to obtain the most desired chemical compounds [26]. Pullulanase appeared to be more efficient than CGTase, the desired products were characterized with higher specificity [28]. In the literature on taste improvement of the bitter–sweet compound stevioside dextrin dextranase (DDase) has also been used [29]. An interesting example can also be formation of C13-O-β-6(2)-β-glucosylsophorosyl-C19-O-β-glucopyranosyl steviol from stevioside by the cultures of Actinomycete [30].

The methods of improvement of steviol glycoside production by breeding

Traditional breeding and selection methods for the most desired traits are currently supported with molecular tools i.e. determination of selective markers combined with specific traits, by polyploidisation, or more radical intervention in the genome and producing transgenic varieties. All these methods contribute to shaping the organism development and improvement both in genetic and commercial terms. Understanding the essence of a given trait and the nature of its inheritance is a powerful tool for further efforts to modify the trait in an organism. Analysis of metabolic paths, determination of the enzymes participating in the paths, methods of regulation and genes responsible for the processes provide important information, which can help develop the desired traits [31].

The most characteristic stevia trait is its ability to biosynthesize steviol glycosides, therefore the breeding of this species is focused on modification of the content and composition of these compounds. Due to the specific taste of stevioside attention is paid to lowering its share in the total steviol glycosides, while for A and C rebaudiosides to their increase as they do not have bitter taste. The highest content of steviol glycosides is found in leaves and thus other modified traits are leaf yields and leaf-stem mass ratio. The studies conducted by Brandle and Rosa [32] showed that economically important breeding traits of stevia are characterized with high variability within populations and high heritability. These traits include leaf yield (h^2=62.1), leaf – stem ratio (h^2 = 78.8) and content of steviol glycoside (h^2=76.6). Due to high heritability they are susceptible to modification by selection [32]. Moreover, the content of stevioside and A rebaudioside are negatively correlated, while contents of A and C rebaudioside are positively correlated. The work published by Brandle [33] showed, as a result of cross breeding plant groups of different profiles of steviol glycoside levels, that the presence or absence of A rebaudioside is controlled by a single dominating gene, while its amounts are regulated by a higher number of loci. The A and C rebaudioside ratio is determined by a single additive gene and these traits undergo co-segregation. The authors suggested that the same enzyme is responsible for synthesis of both

compounds [33]. Those stevia traits make it a plant of high breeding potential and susceptible to improvement by the selection, what allows for obtaining of more efficient varieties.

In case of plants that often self-pollinate and show high differences in a given trait, as it is in stevia, selection is an effective method of obtaining improved varieties. For more than thirty years of breeding stevia, the steviol glycoside levels in leaves has been raised from 2–10% to even 20% of dry mass [31]. Yet, the selection done so far has been based on phenotypic traits, which depend very much on the breeding conditions, and also on the plant age. It is estimated that in case of young seedlings only 20–30% of the variability is determined genetically. Therefore selection should be made on mature plants, what in turn prolongs the time needed before obtaining the results. Moreover, determination of the glycoside content in leaves with HPLC is expensive and time consuming [34]. Biotechnological tools provide opportunities for improvement of the desired stevia traits. *In vitro* tissue cultures are used for massive propagation and/or for obtaining completely haploid genotypes in anther cultures. The identification of molecular genetic markers aims at locating the loci in the stevia genotype that affect biosynthesis of steviol glycosides. It is also important to understand more deeply the biochemical synthesis pathways that take part in steviol glycoside synthesis, what will allow for regulating gene expression. Induced mutations may successfully be used for improvement of the traits with low variability within the population.

So far, few stevia cultivars that are characterized with higher yielding of steviol glycosides became patented. A cultivar is a breeding variety of a plant characterized with specific use or ornamental traits, stability and homogeneity, obtained by the way of breeding treatments such as selection, cross breeding, polyploidisation or mutation induction. Cultivars are most often clones, as maintaining their traits requires limitation of generative reproduction and using vegetative reproduction instead.

An especially useful technique for improvement of quantitative traits in cross-pollinated species with high differentiation of a given trait within the population (e.g. in stevia) is *recurrent selection*. This method involves selection of recombinants showing traits better in comparison with the general population and further cross breeding of the recombinants. As a result a subpopulation forms where alleles determining desired traits occur at higher frequency than in the initial population. The cycle is repeated until significant response is achieved. Stevia breeding lines RSIT 94-1306 and RSIT 751 with improved content of glycosides were obtained with controlled cross breeding and selection method described by Brandle and Rosa (1992) [32]. In 1998 an RSIT 95-166-13line was bred, which is unique due to its C rebaudioside – stevioside ratio [35]. All three cultivars were bred vegetatively.

Development of synthetic and complex cultivars is of special importance in producing such plants as AC Black Bird and PTA – 444 i.e. with altered glycoside content [36, 37]. Some of the cultivars, despite extremely high glycoside production, are self-sterile and can only be bred vegetatively. However, PTA – 444 can

be reproduced with seeds. The necessity of use of vegetative breeding raises the costs of the plant production and limits their commercial usefulness, yet they can be still used for hybrid production [22, 36].

Plant hybrids are created by cross breeding of plants of different species or genus. Intentional, controlled production of hybrids allows for introducing new genes into the gene pool, what contributes to enriching the genetic diversity. This phenomenon is positive for homogenous populations of crops, where it can enhance heterosis. It might also be a methods of breeders to create new varieties of better use characteristics e.g. resistance to diseases or vitality. In 2006 Wang patented a method of breeding stevia hybrid plants [38]. In 2001 Sun suggested a method for obtaining seeds, which employs the technique of producing vegetative hybrids [34]. A vegetative hybrid forms as a result of grafting a part of a plant onto another plant.

Mutation induction is a tool that accelerates considerably the development of plants with desired traits as compared with the traditional breeding methods. The use of mutagens, both physical and chemical, allows for much faster obtaining of genetic diversity within a population. These methods also find application when a given trait shows very low variability within the population. Until now this method has not been used in stevia, as other methods are easily available and efficient.

Polyploidisation is successfully used for improving yields of many crops. Polypoloid plants are often characterized with higher adaptability to environmental conditions and larger organs and cells. The plants produced as a result of cross breeding of specimen with different chromosome numbers are most often either completely or partially sterile. Stevia triploids are obtained by placing seeds in colchicine solution or by cross breeding a tetraploid female plant with a male diploid. Triploidity in stevia is linked with higher content of a rebaudioside and in larger leaves [39], where stevia tetraploids have bigger and thicker leaves, what can potentially lead to increase in biomass yields. All the poliploids also had nonfunctional pollen [40].

Anther cultures, i.e. *in vitro* cultures formed from immature anthers, are used for obtaining haploid plants. They can be used for obtaining double haploids i.e. plants completely homozygotic. Specimen from homozygotic populations in terms of a given trait might be used for hybridization. In case of stevia, plant regeneration from anthers were successfully carried out by Flachsland et al. [41].

Development in use of molecular markers and identification of markers linked with specific traits creates new possibilities and ways of plant breeding. It allows for discovering desired traits earlier on in the plant development by their link to easily detectable molecular marker. This eliminates the need to produce mature plants and shortens considerably the time needed for evaluation of the traits of a given specimen. Moreover, it is possible to run the selection process on smaller populations. Genetic maps for stevia were created with the use of RAPD (Randomly *Amplified Polymorphic DNA*) w technique in 1999 [34]. Constructing genetic maps

will enable to the molecular selection techniques in breeding of stevia based on genetic markers and will lay the grounds for studies on the stevia genome organization and metabolism. The next step in research should include matching specific markers for economically important traits and shaping them, so that they can be easily used in the plant material.

Despite the studies discussed above, stevia remains a species investigated to a rather limited extent. Further works, development of new varieties and adjustment of breeding techniques to the plant will allow for increase of its potential in food industry by improvement of quality and quantity of the yields.

CONCLUSION

The review of available literature clearly indicates growing interest in natural sweeteners, what stimulates the development of obtaining technologies. Stevia, as a natural, low-caloric sweetener might have potential application in human diet as saccharose substitute, the latter being consumed in excessive amounts. The analysis of available information suggests that the *in vitro* growth conditions can significantly influence the biomass yield in stevia and biosynthesis of steviol glycosides. Micropropagation of stevia is the most efficient method of obtaining these plants in industrial scale. Optimization of the *in vitro* culture conditions in the processes of sterilization, propagation and acclimatization is an important element in practical application of this method.

REFERENCES

1. Sivaram L, Mukundan U. *In vitro* culture studies on *Stevia rebaudiana*. Vitro Cell Dev Biol-Plant 2003; 39(5):520-523.
2. Tamura Y, Nakamura S, Fukui H, Tabata M. Comparison of stevia plants grown from seeds, cuttings and stem-tip cultures for growth and sweet diterpene glucosides. Plant Cell Rep 1984; 3(5):180-182.
3. Thiyagarajan M, Venkatachalam P. Large scale *in vitro* propagation of *Stevia rebaudiana* (bert) for commercial application: Pharmaceutically important and antidiabetic medicinal herb. Ind. Crops Prod 2012; 37(1):111-117.
4. Kinghorn AD. Biologically active compounds from plants with reputed medicinal and sweetening properties. J Nat Prod 1987; 50(6):1009-1024.
5. Brusick DJ. A critical review of the genetic toxicity of steviol and steviol glycosides. Food Chem Toxicol 2008; 46(7):S83-S91.
6. Ibrahim IA, Nasr MI, Mohammed BR, El-Zefzafi MM. Nutrient factors affecting *in vitro* cultivation of *Stevia rebaudiana*. Sugar Tech 2008; 10(3):248-253.
7. Joint FAO/WHO Expert Committee on Food Additives, Meeting WHO, Food and Agriculture Organization of the United Nations. Compendium of food additive specifications. Addendum 12. World Health Organization: Food and Agriculture Organization of the United Nations; 2004.
8. Carneiro JWP, Muniz AS, Guedes TA. Greenhouse bedding plant production of *Stevia rebaudiana* (Bert) Bertoni. Can J Plant Sci 1997; 77(3):473-474.
9. Hassanen SA, Rasha MA. Khalil. Biotechnological studies for improving of Stevia (*Stevia rebaudiana* Bertoni) *in vitro* plantlets. Middle-East J Sci Res 2013, 14 (1):93-106.

10. Jain P, Kachhwaha S, KothariInt SL. Biotechnology and metabolic engineering of Stevia rebaudiana (Bert.) Bertoni: perspective and possibilities. J Life Sci Bt & Pharm Res 2014; 3(2):15-37.
11. Bondarev N, Reshetnyak O, Nosov A. Effects of nutrient medium composition on development of *Stevia rebaudiana* shoots cultivated in the roller bioreactor and their production of steviol glycosides. Plant Sci 2003;165(4):845-850.
12. Jain P, Kachhwaha S, Kothari SL. Optimization of micronutrients for the improvement of *in vitro* plant regeneration of *Stevia rebaudiana* (Bert.) Bertoni Indian J Biotechnol 2012; 11:486-490.
13. Jain P, Kachhwaha S, Kothari SL. Improved micropropagation protocol and enhancement in biomass and chlorophyll content in *Stevia rebaudiana* (Bert.) Bertoni by using high copper levels in the culture medium. Sci Hortic 2009; 119(3):315-319.
14. Bondarev N, Reshetnyak O, Nosov A. Peculiarities of diterpenoid steviol glycoside production in *in vitro* cultures of *Stevia rebaudiana* Bertoni. Plant Sci 2001; 161(1):155-163.
15. Taware AS, Mukadam DS, Chavan AM, Taware SD. Comparative studies of *in vitro* and *in vivo* grown plants and callus of *Stevia rebaudiana* (Bertoni). Int J Integr Biol 2010; 9(1):10-15.
16. Anbazhagan M, Kalpana M, Rajendran R, Natarajan V, Dhanave D. *In vitro* production of *Stevia rebaudiana* Bertoni. Emir J Food Agric 2010; 22(3):216-222.
17. Yadav AK, Singh S, Dhyani D, Ahuja PS. A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)]. Can J Plant Sci 2011; 91(1):1-27.
18. Dey A, Kundu S, Bandyopadhyay A, Bhattacharjee A. Efficient micropropagation and chlorocholine chloride induced stevioside production of *Stevia rebaudiana* Bertoni. C R Biol 2013; 336(1):17-28.
19. Mathur S, Shekhawat GS. Establishment and characterization of *Stevia rebaudiana* (Bertoni) cell suspension culture: an *in vitro* approach for production of stevioside. Acta Physiol Plant 2013; 35(3):1-9.
20. Madan S, Ahmad S, Singh GN, Kohli K, Kumar Y, Singh R, Garg M. Stevia rebaudiana (Bert.) Bertoni – a review. Indian J Nat Prod Res 2010ł 1(3):267-286.
21. Bondarev N, Nosov A, Kornienko A. Influence of several cultural factors on the growth and efficiency of Stevia callus and suspension cultures. Biotechnology 1997; 7(8):30-37.
22. Lee KR., Park JR, Choi BS. Studies on the callus culture of stevia as a new sweetening source and the formation of stevioside. Korean J Food Sci Technol 1982; 14(2): 179-183.
23. Swanson SM, Mahady GB, Beecher CWW. Stevioside biosynthesis by callus, root, shoot and rooted-shoot cultures *in vitro*. Plant Cell Tissue Organ Cult 1992; 28(2):151-157.
24. Hsing YI, Su WF, Chang WC. Accumulation of stevioside and rebaudioside A in callus culture of *Stevia rebaudiana* Bertoni Bot Bull Acad Sin 1983; 24:115-119.
25. Richman A, Swanson A, Humphrey T, Chapman R, McGarvey B, Pocs R, Brandle J. Functional genomics uncovers three glucosyltransferases involved in the synthesis of the major sweet glucosides of Stevia rebaudiana. Plant J 2005; 41(1):56-67.
26. Kinghorn AD. Stevia. The Genus Stevia [on-line]. Londyn: Taylor & Francis; 2002.
27. DuBois GE, Stephenson RA. Diterpenoid sweeteners. Synthesis and sensory evaluation of stevioside analogs with improved organoleptic properties. J Med Chem 1985; 28(1):93-98.
28. Lobov SV, Kasai R, Ohtani K. Enzymic Production of Sweet Stevioside Derivatives: Transglucosylation by Glucosidases. Agric Biol Chem 1991; 55(12):2959-2965.
29. Yamamoto K, Yoshikawa K, Okada S. Effective production of glycosyl-steviosides by α-1,6 transglucosylation of dextrin dextranase. Biosci Biotechnol Biochem 1994; 58:1657-1661.
30. Kusakabe I, Watanabe S, Morita R, Terahara M, Murakami K. Formation of a transfer product from stevioside by the cultures of Actinomycete. Biosci Biotech Biochem 1992; 56:233-237.
31. Yadav SK, Guleria P. Steviol Glycosides from stevia: biosynthesis pathway review and their application in foods and medicine. Crit Rev Food Sci Nutr 2012; 52:988-998.
32. Brandle JE, Rosa N. Heritability for yield, leaf:stem ratio and stevioside content estimated from a landrace cultivar of *Stevia rebaudiana*. Can J Plant Sci 1992; 72(4):1263 1266.
33. Brandle J. Genetic control of rebaudioside A and C concentration in leaves of the sweet herb, *Stevia rebaudiana*. Can J Plant Sci 1999; 79(1):85-91.
34. Sun J. Method for breeding hybridized seed of *Stevia rebaudinum* [on-line]. China (SIPO) Patent no.CN1237720-A. 2001.
35. Brandle J, Sys EA, Marsolais AA. Stevia plant named `RSIT 95-166-13` [on-line]. US Patent no. US PP10563 P; 1998.

36. Brandle J. Stevia rebaudiana with altered steviol glycoside composition [on-line]. US Patent no. US 6255557 B1; 2001.
37. Morita T, Bu Y. Variety of Stevia rebaudiana Bertoni [on-line]. US Patent no. US 6031157 A; 2000.
38. Wang Q. Systemic asexual stevia parent selection breeding process for producing hybrid [on-line]. China (SIPO) Patent no. CN 1985575 B. 2007.
39. Shuichi H, Tsuneo Y, Satoshi F. Breeding of tyriploid plants of stevia (*Stevia rebaudiana* Bertoni) with high rebaudioside A content. Jpn J Trop Agric 2001; 45(4):281-289.
40. Oliveira V. de, Forni-Martins ER, Magalhães PM, Alves MN. Chromosomal and morphological studies of diploid and polyploid cytotypes of *Stevia rebaudiana* (Bertoni) Bertoni (Eupatorieae, Asteraceae). Genet Mol Biol 2004; 27(2):215-222.
41. Flachsland E, Mroginski L, Davina J. Regeneration of plants from anthers of *Stevia rebaudiana* Bertoni (Compositae) cultivated *in vitro*. Biocell 1996; 20:87.

ZASTOSOWANIE KULTUR IN VITRO STEWII (*STEVIA REBAUDIANA* BERTONI) DO UZYSKANIA MATERIAŁU BOGATEGO W GLIKOZYDY STEWIOLU

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

Stewia jest rośliną budzącą szerokie zainteresowanie ze względu na zdolność syntezy grupy związków chemicznych o słodkim smaku – glikozydów stewiolu. Glikozydy stewiolu są z powodzeniem stosowane jako naturalny słodzik, a niektóre z nich wykazują również właściwości terapeutyczne. W pracy przedstawiono dostępne informacje na temat wykorzystana roślinnych kultur tkankowych stewii ze szczególnym uwzględnieniem możliwości ich użycia dla potrzeb przemysłu spożywczego. Szeroko rozeznano i przeanalizowano badania z użyciem technik *in vitro* w hodowli stewii i możliwości ich wykorzystania w procesie biosyntezy ważnych dla przemysłu spożywczego metabolitów wtórnych. Uwzględniając zarówno dotychczasowe osiągnięcia, jak również najnowsze publikacje dotyczące tematyki stewii, oceniono możliwość praktycznego zastosowania omawianych technik, jak również potencjalne możliwości ich przyszłego rozwoju.

Słowa kluczowe: *glikozydy stewiolu, kultury in vitro, metabolity wtórne, mikropropagacja, Stevia rebaudiana, stewia, stewiozyd*