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Development in the Production of Natural Sweetener (*Stevia rebaudiana*) in Bulgaria

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Abstract: It's hard to imagine life of modern mankind without sugar plants. Sugar beet, sugar cane and other less known plants have been for a long time a source for obtaining sugar for the food industry. Recently ever more attention is being paid to sweet-tasting plants without containing sugar. This is because not all people can use sugar and furthermore non- sugar sweeteners create a sweet taste at much lower concentrations. Therefore hunger for sweetness had led man to find alternative forms of intense sweeteners which can offer sweet taste to the consumers without calories. There are sugar substitutes (natural and artificial) that have virtually no calories. Not accidentally stevia is called honey grass - it is the sweetest plant on the planet. In its natural form stevia is much sweeter than sugar and at present it is considered for the best natural sweetener and sugar substitute. Many countries conduct researches in order to detect natural, low-calorie sweeteners of plant origin which are not harmful for human body. Various climatic conditions impose specific studies about: obtaining propagating material; selection of suitable soil and climatic conditions for growing; irrigation norms; fertilization; establishment favorable terms for gathering, drying and storing of yield obtained of foliage. Aim of this report a part of development of technology for cultivation of Stevia in Central Bulgaria, Plovdiv region is, as a source of natural sweeteners. Considered is assessment of productive and economic qualities of obtained populations under field conditions. Studied material from Stevia are contrasting on studied parameters. Plant parameters including height of the whole plant; height of main stems; height of additional (spurs) stems; number of basic (central) stems; number of additional stems; total weight of stems; weight of whole plant; weight of green (raw) leaves; weight of absolutely dry mass of leaves and weight of root were analyzed. Biometric measurements show that crop formation, leaves productivity and architectonics of plants are the highest in the region of Plovdiv. Around 80 % of required biomass of dry leaves is realized. The reduced height of the plant leads to improved profitability of the stem. In applied method of cultivation Stevia reaches to formation of reproductive organs too - Flowers and Seeds.

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1. Introduction

Growing awareness among people about the negative effects of refined sugar and its substitutes has changed dramatically sweeteners market in the last few decades. There were sugar substitutes [natural and synthetic] that contains virtually no calories. Unfortunately, many of them posses poor nutritious value and are not harmless to human health, as is the case with aspartame, also known by names such as NutraSweet and E 951 [approved by the FDA in 1981]. Intensive intake of such products have proved to be carcinogenic or linked with the spread of variety of diseases or physiological disorders (Keerthi et al., 2011).

Many countries are conducting surveys to detect natural, low-calorie sweeteners of plant origin, which are not harmful to the human body. Following numerous scientific literature on the subject, the extract from the leaves of the South American stevia plant [Stevia rebaudiana B.], remains unsurpassed sugar substitute. Sensory and functional properties of Stevia leaves are superior to available high-potency sweetners. There are no evidence of toxicity or mutagenicity of Stevia's components as well as any side effects cause by its usage. Based on these properties Stevia is potential contender to become a major source of natural sweetner for growing dietary market (Goyal et al., 2010)

Diterpene glycosides present in leaves are responsible for pronounced sweet taste. Currently more than 100 compounds including Steviol, stevioside and rebaudioside (A, B, C, D, E, F), steviol bioside, dihydroisosteviol and dulcoside-A are extracted from Stevia plant (Wölwer, 2012; Gupta et al., 2013). In addition to its sweetness, stevia is blessed with significant nutritional and chemical composition i.e., it contains amino acids, minerals and phytochemicals, especially polyphenols that significantly increase antioxidant activity of Stevia. Plant components like leafs and leaf powder from stevia acts as potential source of antioxidant activity in low calorie foods (Singh and Rao, 2012; Rao et al., 2014).

Sweetening compounds found in stevia are of vital importance as they are highly soluble in water, contains high sweetness with low calories and are stable in acidic conditions. These compounds are extracted from Stevia leaves and are commercially available and widely used for sweetening a wide range of foods in various countries (Sharoba et al., 2012). E960 European Union Regulation No. 1129/2011, with acceptable daily intake (ADI) of 4-7.9 mg kg^{-1} human body weight. (Carocho et al., 2014; Gupta et al., 2013)

Cultivation of Stevia became inevitable alternative to sugar especially for 347 million diabetic population across the world (WHO, 2015). Following the source of information for growing stevia a major advantage can be brought - its wide range of dissemination that allowed to be introduced in different countries.

There are numerous reports that are increasing interest and demand towards this plant. There are general date for its cultivation and usage (Jeffrey Goettemoeller and Karen Lucke, 2006; Deepak Acharya. 2008; Hossain M.A. 2008.), its advantages over artificial sweeteners such as aspartame, saccharin, acesulfame-K etc. (Stewart K., 2008). It was found that stevia and stevia extract are essential products for patients with diabetes, obesity, diseases related to metabolism.

The diffrent climatic conditions require specific developments regarding: obtaining of planting material, selection of suitable soil and climatic conditions for cultivation, irrigation norms; fertilization; harvest with favorable terms, drying and storage of foliage yield.

Along with efforts to prove its safety and utility to be used use as food or dietary supplement, another research is carried out for the development of cultivation technologies in Europe (Christa Lankes/Ralph Pude 2008; Sumida T., 1968).

Stevia has been studied since the 80's, when invitro and invivo methods for propagation and breeding technology had been developed, which enabled to be recovered and expanded the researches of the promising culture.

Until 2008 more than 20 countries (Japan, China, Korea, India, Taiwan, Brazil, Russia, Switzerland, etc.) use stevia as a food additive and sweetener. This is the main reason to be restored and developed the work of this plant and its promotion and cultivation in the region of Plovdiv, Bulgaria.

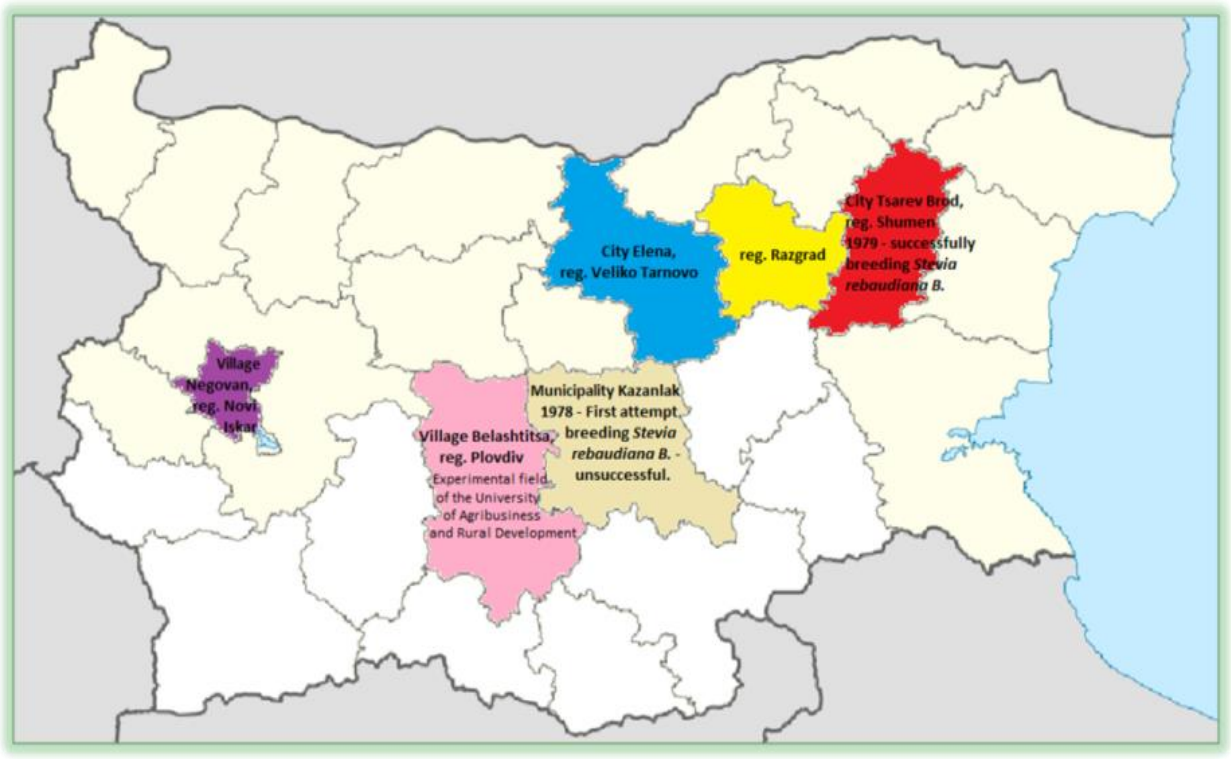

Figure.1 Areas in Bulgaria with partly stevia cultivation

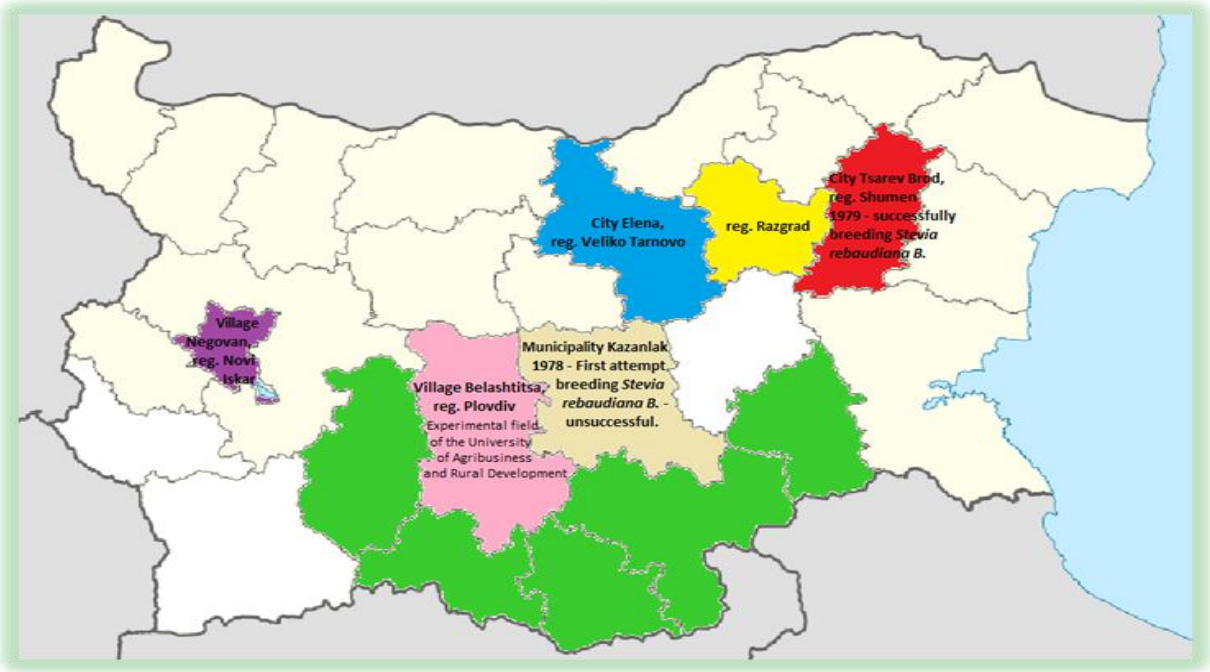

Figure 2. Increasing areas under field cultivation of stevia.

The main objective of the report is a survey and evaluation of the production potential of Steviarebaudiana B. in Bulgaria as well as reporting features of production, resulting in its cultivation in the region of Plovdiv. The report is a part of technology development for Stevia cultivation as a source of natural sweetener and as an alternative crop for the Eastern Rhodopes. To realize this goal several tasks are placed: assessment of productive and economic properties from a field-derived populations and development of some elements of agrotechnics for growing.

1.1 Soil and Climatic Conditions in Bulgaria

Stevia prefers a sandy soil, requiring a warm sunny condition. Semi-humid subtropical climatic conditions with temperature extremes ranging between 21 to 43°C with an average temperature of 24°C favors plant growth (Huxley, 1992). Stevia requires frost free period for its growth, as during winter (Ramesh et al., 2007). Climatic conditions prevailing in Bulgaria (except winter) favors *Stevia rebaudiana* cultivation with good quantitative and qualitative indicators, including high leaf yield and steviosides content. Although Bulgaria is small (area: 110 912 km^2) in size, but in terms of climate and soil suitable for cultivation of Stevia. It is characterized by:

- Wide variety of climatic, geological, topographic, and hydrologic conditions
- Temperate zone, with an average altitude of 470 m
- Typical continental and changeable climate in spring and hot and humid summers and moderate temperatures around 28-30 ° C
- 55-80% relative humidity and rainfall of 450-750 mm.
- Soils are referred to as neutral, richest in organic and mineral substances; have good water holding capacity and air permeability.

1.2 History of *Stevia Rebaudiana* in Bulgaria

In Bulgaria Stevia comes from Japan more than 30 years ago as a small plant in a test tube and its cultivation continues until today, which is at a relatively early stage of development.

Originally the Stevia plant was taken to the Institute of Kazanlak but after unsuccessful attempts for its propagation, few surviving seedlings of stevia were brought in the Institute of Shumen in 1979. After months of work in the Tissue Culture Laboratory of the Institute their efforts are being rewarded. Thus in 1980 the Institute has plants, which were vegetative propagated in test tubes (in vitro) and until 1990 the scientific team had been working on research projects and reproduction tasks, adaptation to external conditions, until they started with plant breeding (Varbanov et al., 1996)

Figure 3. Height of the plant in Bulgaria A) sowing of plants in Shumen region; B) sowing of plants in Plovdiv region; C) whole plant of Shumen region; D) whole plant of Plovdiv region.


Figure 4. Stevia – branches.

Economic crisis after transition in our country forced to stop working with this exceptional herb for a while. Until five years ago, when it began again and the plant was first presented at "AGRA" exhibition in Plovdiv. By then the herb was maintained in vitro. Currently a major research project is developing in the field of selection, introduction and cultivation technology of stevia. Great diversity of genetic material make scientists optimistic about viability of the plant in our country. Total amount of stevia glycosides of local selection materials is from 110.4 to 149.8 mg/g dry weight, which is a relatively good content. Unfortunately the plant is not widely grown in our country. Areas where the unique herb is partly cultivated are districts of Shoumen, Razgrad, Veliko Tarnovo, Plovdiv and Sofia – the region of Novi Iskar (Fig. 1, 2).

1.3 Biological Conditions and Technology of Stevia Cultivation in Bulgaria

The root system of stevia plant is well branched (Fig. 3, 4) and developed (Fig. 5, 6). For its belonging to *Asteraceae* family, in contrast to other tropical and subtropical plants, stevia can be cultivated in wide range of soils and climatic zones.

Flowering occurs in July till September. In our climatic conditions a small amount of plants reaches flowering, but main aim of the breeding work is the creation of genotypes with more biomass (Fig. 5). Under existing conditions in Bulgaria to collect seeds was difficult, because the sweet herb is a short- day plant so it starts to flower in the autumn, therefore seeds rarely ripen.


Figure 5.Stevia flowering in different locations of Bulgaria.

Figure 6. Root system of plant, cultivated in Bulgaria (a) 2 months old plant (b) annual's root (c) biennial's root.

Figure. 7. Seeds stevia obtained from different agro-climatic zones of Bulgaria.

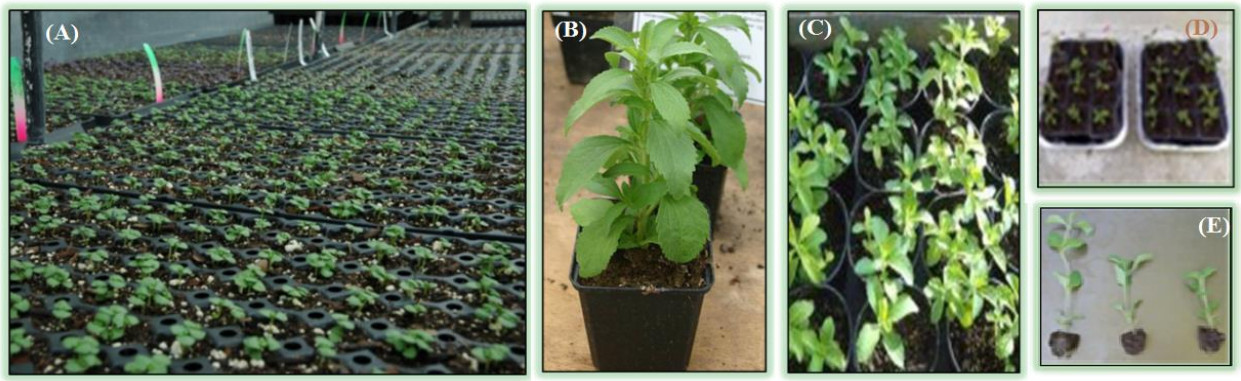

Figure 8.Stevia Seedlings Production.

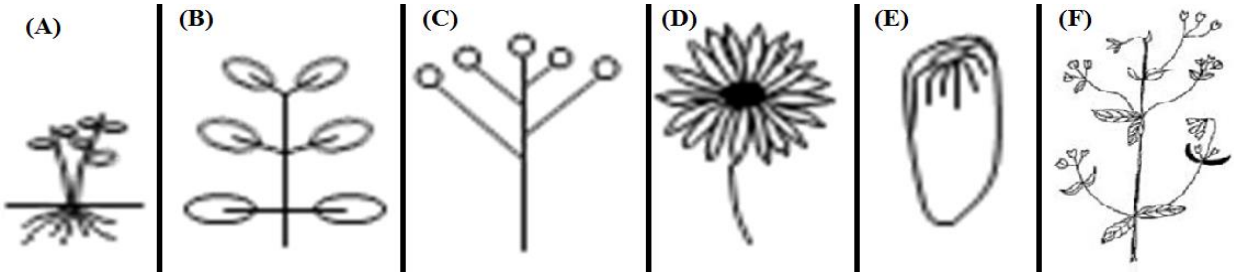

Figure 9. Organography of Stevia a) formation of seedlings from the root; b) forming a leaves; c) formation of flower buds; d) flowering; e) appearance of seeds; f) whole plant.

Plants left to form seeds lose significant part of their beneficial qualities due their late harvest. Often seeds which are collected in Bulgaria are unripened and have low capability of germination (Fig. 7). Other studies also reported poor germination rate of Stevia seeds (10-50%) and smaller seed size (0.2 - 0.3 g thousand-seed weight) which restrict its propagation through seeds. Therefore alternative propagation methods are commonly used for Stevia cultivation (Sakaguchi and Kan, 1992; Goettemoeller and Ching, 1999; Yadav et al., 2011; Kumar et al., 2014).

In Bulgaria Stevia is an annual plant but with a perennial rootstock use. Stevia is grown by seedlings (seeds or vegetative), then two months old plants are planted outdoors with south or southwest exposures. Stevia is low temperature susceptible and needs frost free conditions for its growth (Huxley, 1992; Ramesh et al., 2007), which necessitates to be grown from seedlings for local conditions of country (Fig. 8). Getting seedlings from seeds is usually used in selection activities for obtaining new plant varieties (Fig. 9).

2. Methods of Stevia propagation

Preferred methods for plants propagation in Bulgaria are:

2.1 Vegetative propagation by tissue culture (in vitro)

Tissue Culture Laboratory of the Agricultural Institute in Shumen has developed a technology for vegetative multiplication of stevia on in vitro conditions. This method allows for short period of time from selected plants to be obtained more plants for necessity of the production (Fig. 10).

2.3 Vegetative propagation by green stem cuttings taken from root suckers

Mid-May to early June is the best time to take cuttings. Cuttings with three or five internodes are cut from the uterine plant by leaving leaves only in the top. They are placed deeply in substrate. Then they are transplanted into a pot and 30-45 days later when the seedlings reach 10-12 cm height, the plants are put into the ground (Fig. 11).

2.3 Vegetative propagation by division of root and field planting

Stevia rhizomes were kept under strict temperature regime - not more than 8^{o}C (otherwise they begin to sprout) and not less than 4^{o}C (because they freeze), and 80-85% humidity. They were stored in a basement or in a room without lights. Drying and waterlogging were not allowed. In the spring rhizomes were ready for repeated cultivation after all danger of frost is past.

During the next season by planting the rhizomes stevia growth significantly accelerated as compared to plants grown from seeds. Second year after planting, strong root system is formed in the soil and each plant gives 10-15 suckers. The rhizomes are divided a month before planting. They are carefully cut with sharp knife and the parts must have 1-2 live leaf-buds. When use stevia for field production, it is better to plant the crop in the spring after the danger first frosts has passed. Mandatory conditions for its normal growth are enough light and regular watering. During the autumn soil was dig and dressed with 3-4 kg farmyard manure and 50 g m^{-2} complex mineral fertilizers. Seedlings were planted in field during first week of June and 1-2 weeks earlier in greenhouses -. In the holes (5-6 cm deep) bio-humus and wood ash were added. The planting distance was kept 60 x 20 cm and 70 x 30 cm. In hot days plants were overshadowed. Soil was kept moist and standard management practices were adapted to keep the field weed free. In dry periods and after cutting stevia irrigation was applied.

Figure 10. Ex vitro adaptation (A) of Stevia plants produced through tissue culture.

Figure 11. Green cuttings of Stevia.

Figure 12 Root system of Stevia plants, cultivated in different agro-climatic regions of Bulgaria.

Figure 13 Process of Stevia harvesting

Figure 14. Dehydrating process and packing of dry leaves of Stevia.

After rain or watering heavy soils was carefully loosen up to a depth of 3-4 cm, to avoid any physical damage to the plants. Mineral or organic fertilizers were periodically applied to Stevia as per recommendations (Fig. 11).

Stevia looks like nettle and it can be harvested by mowing at least 2-3 times per year. But first cuts have lower levels of glycosides, account for its incredible sweetness. In field production it is well to be harvested in autumn when leaves and stems have high content of carbohydrate substances. The best time to harvest stevia is right before the plant starts to flower, because at this moment maximum sweet substances are accumulated. When plants reach their maximum

size, they are cut up to 1/3 of the plant's height while leaving leaves at the bottom parts of the shrub for further growth (Fig.12).

Leaves should not be singly trimmed, because bare branches are drying. Stevia leaves are cut and then should be dried. For good storage, the moisture content of dried 'stevia' should be less than 10%. Until the end of October another crop is gathered, and then rhizomes are harvested. Stevia leaves are cut and then should be dried.

3. Optimizing Process Parameters for Stevia Field Cultivation in Bulgaria

Five years agronomic studies of *Stevia rebaudiana*, grown in laboratory and field conditions, show that the yield of the leaf mass has increased over the last two industrial years. By improving the technology yield of dry leaf of 50-70 g per plant is achieved, which supposed yield of dry leaf from 2500 to 3000 kg/ha. (Fig.13)

Growing of Stevia under conditions in Bulgaria, pest infestation is minimal and the damage is mainly in seedling production, caused by different caterpillars of *Lepidoptera* order, *Noctuidae* genus. Significant damages to the first leaves are caused by *Septoria steviae* and *Sclerotinia sclerotiorum*. Good agricultural practice can control pests, diseases and weeds.

Due to soil and climatic conditions in Bulgaria, Stevia is characterized by different productive potential (Table 1). Plants are harvested at the end of September and they are 80-120 cm tall, compared to those under natural conditions which height is 60-80 cm. In the table are shown parameters surveyed in the two regions the study their mean values, standard deviation and coefficient of variation. The variation in the values of the coefficient for the studied parameters show a great variability from 1 to 50% in the region of Shumen, and 1 to 35% in the region of Plovdiv.

Biometric measurements in the table show that crop formation, leaves productivity and architectonics of plants are the highest in the region of Plovdiv, whereas species in Shumen the ratio yield of leaf and stems is 75% and 86%, due to strong branching and rapid budding and flowering. (Fig.15).

It is interesting to note that in both regions the trend of ratio leaves to the whole plant is the same, which indicates that during cultivation in subsequent years would be observed extension of leaf production and proportion between leaves yield to the whole plant. After the third year, the trend is to be produced larger number of new stems and leaves from the whole plant, and thus their ratio will be reduced (Fig.16a).

Weight of leaves about 3000 kg ha^{-1} with a steviosides concentration 150 mg g^{-1} determine yield about 450 kg ha^{-1}. If steviosides are 210 times sweeter than sucrose, than the steviosides yields are equivalent to 94.5 t ha^{-1} of sugar (Fig.16b).

During the studies, using Principal Component Analysis (PCA) of the relationship between indicators that forms Stevia, it was found that in the Shumen region total biomass production from 2 400 kg/ ha is possible from 17% roots, 37% stems and 46% leaves. The trends in the Plovdiv region are the same where biomass yield is 3 000 kg/ha from 15% roots, 36% stems and 49% leaves. (Fig.17).

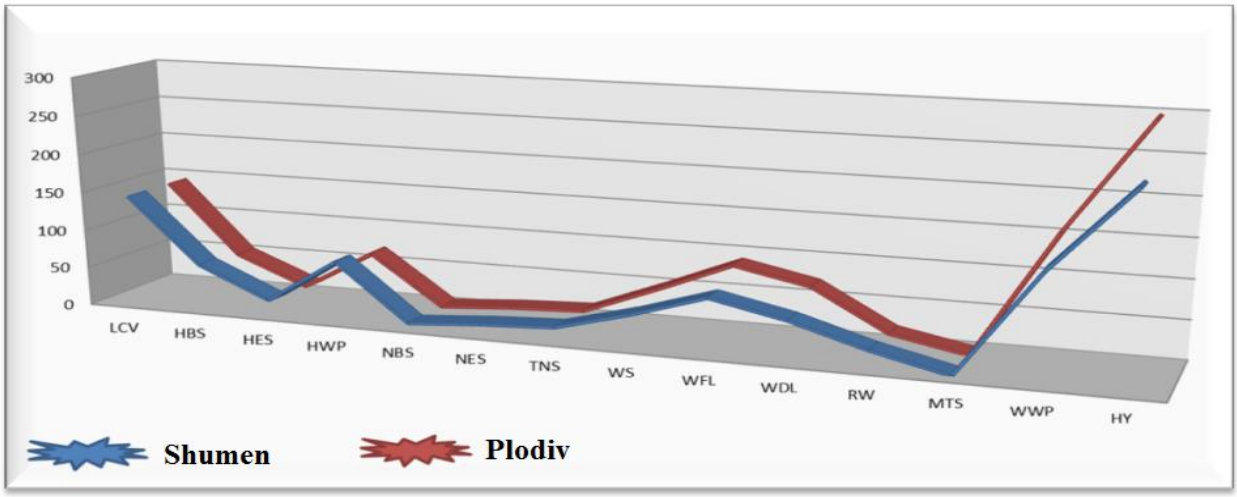

Figure 15. Parameters phenological stages of stevia.

Table 1. Parameters characterizing phenological stages of stevia in two agro-ecological zones of Bulgaria

Parameters	Shumen Region			Plovdiv Region		
	x ± Se	sd	VC,%	x ± Se	sd	VC,%
Length of the cycle of vegetation (days) (LCV)	142,92 ± 0,89	3,98	17,24	143,12 ± 0,85	3,81	15,50
Height of basic stem (cm) (HBS)	58,21 ± 3,47	7,76	16,11	52,02 ± 4,16	9,32	22,18
Height of extra stem (cm) (HES)	18,80 ± 3,44	7,69	40,09	15,89 ± 2,17	4,86	30,62
Height of the whole plant (cm) (HWP)	77,01 ± 6,60	14,76	18,96	67,91 ± 5,41	12,11	20,59
Number of basic stem (NBS)	4,52 ± 0,54	1,20	26,72	3,94 ± 0,34	0,77	19,75
Number of extra stem (NES)	11,50 ± 1,83	4,09	35,46	10,52 ± 0,76	1,70	16,15
Number of stems from plant (TNS)	16,02 ± 0,18	1,11	19,58	14,46 ± 0,24	0,89	14,99
Weight of fresh leaf (g) (WFL)	69,86 ± 6,02	13,46	25,47	89,25 ± 11,10	16,82	27,81
Weight of dry mass leaf (g) (WDL)	50,0 ± 0,34	1,45	19,75	70,0 ± 0,85	4,04	19,38
Weight of the stems from plant (g) (WS)	40,53 ± 9,21	20,60	50,08	50,74 ± 7,96	7,81	35,09
Root weight from plant (g) (RW)	22,12 ± 1,21	2,72	14,59	18,66 ± 2,17	4,86	22,00
Weight of the whole plant (g) (WWP)	132,51 ± 4,75	13,98	36,25	158,65 ± 7,03	8,10	27,55
Mass of 1000-seed (g) (MTS)	0,10 ± 0,14	1,24	9,55	0,23 ± 0,15	1,04	3,38
Stevioside, mg/g of dry mass leaf	110,4 ± 0,52	1,78	18,12	149,8 ± 1,18	2,73	20,60
Leaf : Stem ratio	1,14 ± 0,15	1,00	15,55	0,79 ± 0,11	0,99	13,08
Leaf : Whole plant ratio	0,92 ± 0,12	0,65	1,45	0,98 ± 0,12	0,45	1,08

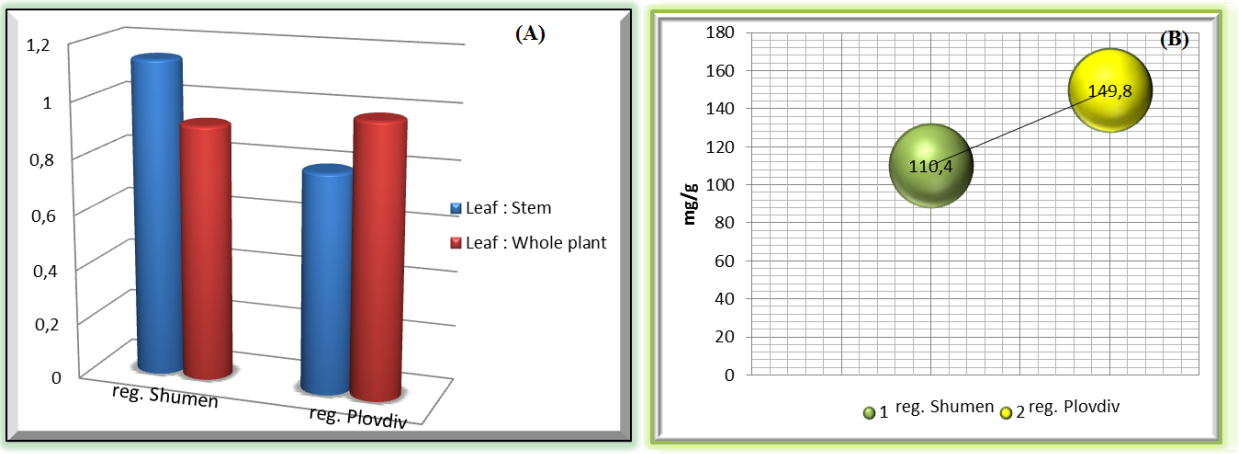

Figure 16. Ratio leaves to the whole plant (a) and steviosides concentration (b) in Stevia grown at different location in Bulgaria.

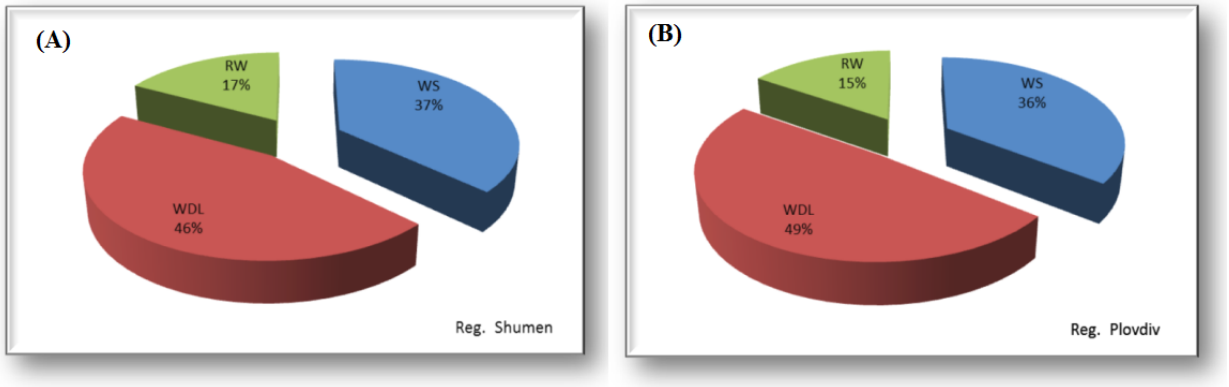

Figure 17. PCA of the relationship between indicators that forms Stevia.

Table 2 Granulated Sugar to Stevia Conversion Chart

Granulated sugar	Dry Leaf of Stevia	Extract of Stevia
1 teaspoon	1/8 teaspoon	On top of a spoon
1 soup spoon	3/8 teaspoon	½ pinch
¼ cup	1 ½ teaspoon	1 pinch
½ cup	1 teaspoon	1/8 teaspoon
1 cup	2 teaspoon	¼ teaspoon
1.7 kg	0.201 kg	0.008 kg
4.5 kg	0.537 kg	0.022kg

The results show that the yield of leaves will grow proportionally with increasing the plants' density in the first year of production. These primary results also show that the improvement of Stevia through selection is possible and that there is a great potential for its production in the southern part of Bulgaria. A large is the difference in the application of sugar and stevia, such as 5 kg of sugar is equivalent to 54 g dry Stevia leaves and 22 milligrams of extract of Stevia.

Plant varies in Steviol glycosides [Stevioside and Rebaudioside A (P-A)] contents from 10 – 15%, as some are reaching to 20% of the dry matter (dried leaves). Therefore breeders strive to produce varieties with higher content of P-A. The ratio between Stevioside and P-A in wild plants is 2.5:1, while in the new varieties this proportion turns to 10:1 to the advantage to unfettered one. (Fig.18). The SGs content in the vegetative organs is presented at the end of the vegetative phase while that in the reproductive organs (flowers and seeds) at the appropriate phase of ontogeny.

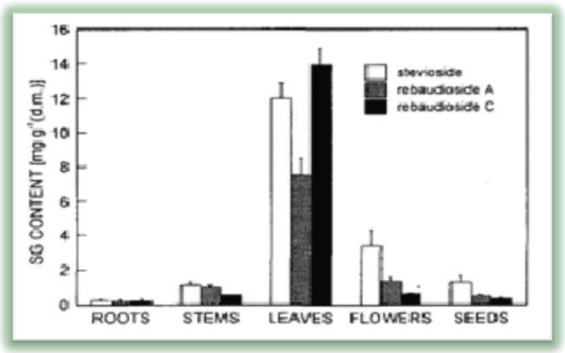

Figure 18. The composition and content of the Stevio glycosides (SGs) in vegetative and reproductive organs.

Dry leaves and stems of the plant are roughly 20 times sweeter than sugar. In the table are shown tentative proportions by replacing sugar with dry, powdered or finely crushed leaves and stevia extract, from plants cultivated in Bulgaria (Table 2).

5. Conclusion

Studies on plants of *Stevia rebaudiana* species show good results and confirm growing and strengthening its position as an alternative crop for the country. It is also appropriate for conditions of Rhodope mountain massif. It is determined that crop development, production and architectonics of plants are the highest in the region of Plovdiv compared to Shumen region, due to better soil and climatic conditions. The study shows that in the region of Plovdiv the greatest influence on yield occupy leaves. Studies are carried out continuously in relation to various climatic conditions, about plant adaptability and fertility in Bulgaria, as well as parallel proving of its safety and utility when using it as food or a dietary supplement.

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