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In Vitro Multiplication of *Stevia rebaudiana* (Bertoni) Genotypes by Using Different Explants

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Abstract: *Stevia rebaudiana* Bertoni that is a member of the Compositae family is one of the most valuable tropical medicinal plants. The origin of *Stevia* is South America, where it can be seen as a wild plant. Further it could be found in semi-arid habitat ranging from grassland to scrub forest to mountain terrain. Conventional cultivation or propagation methods of *Stevia* are as well known for time consuming, unpredictable, unreliable and less productive. Therefore, there is a crucial need to develop methods for rapid multiplication of this valuable plant. Plantlet produced through stem cutting has unstability, however the micropropagation of *Stevia* which may overcome some limitations associated with conventional method can be used for a rapid multiplication. Accordingly, the study results showed that different explants such as leaf, stem and root transferred to different tissue culture media for *in vitro* multiplication of two *Stevia rebaudiana* (Bertoni) genotypes could be reproduced by using different explants.

Key words: Stevia rebaudiana, tissue culture, multiplication, *in vitro*, genotype, explant

INTRODUCTION

The sweet plant *Stevia rebaudiana* Bertoni is native to Paraguay and widespread in this region. The natural habitat of *Stevia rebaudiana* is subtropical grasslands (mesothermalhumid climatic zone) of the mountain range of North-Eastern Paraguay at altitudes of about 200–600 m above sea level, in the Amambay Cordillera (Katayama et al., 1976). As mentioned by Kinghorn (2002) it usually grows in semi-dry mountainous terrains, and its habitat ranges from grasslands, scrub forests, forested mountain slopes and conifer forests to subalpine vegetation.

Stevia (*S. rebaudiana* Bertoni) is a non-caloric natural-source alternative to artificially produced sugar substitutes. The sweet compounds pass through the digestive process without chemically breaking down, making stevia safe for those who need to control their blood sugar level (Strauss, 1995). There have been no reports investigating adverse effects from the use of stevia products by humans (Brandle and Rosa, 1992).

Germplasm of *Stevia* exhibits very poor germination (Debnath et al., 2008). Moreover, population raised from seeds resulted in variability in the major characteristic of Stevia i.e., Stevioside and its percentage. Vegetative propagation is very low in number of individuals that can be obtained from single plant (Yang et al., 1981). Keeping these difficulties in mind; tissue culture is the only technique through which one can obtain mass propagation of *Stevia* plants with homogeneous population. Protocols for propagation of sweet herb from leaf, nodal and axillary shoot explants are established (Yang et al., 1981; Lu, 1993). Ghuari et al. (2009) reported the micro propagation from apical meristem and nodal segment.

Recent reports have shown that plant population produced by direct organogenesis from shoot meristem and leaf explants are homogenous (Tamura et al., 1984a; Miyagawa and Fujioka, 1986). Therefore, genetically identical plants could be provided via regeneration in large scales.

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Afterward, a range of further experiments on stevia tissue culture are carried out such as application of shoot primordial explants (Motomu et al., 1994), shoot apex, nodal and leaf explants (Sivaram & Mukundan, 2003), nodal explants (Rafiq et al., 2007), leaf direct organogenesis (Sreedhar et al., 2008), nodal explants (Ahmed and Salahin, 2007; Mousumi, 2008), *Stevia* rhizogenesis (Tamura et al., 1984b; Ferreria and Handro (1988), rooting (Sivaram and Mukondan, 2003) and duse of diffrent plant hormones (Rafiq et al., 2007; Ahmed and Salahin, 2007; Sreedhar et al., 2008; Ibrahim et al., 2008 and Ibrahim et al., 2008) But, Pourvi et al. (2009) has shown MS medium with 0.5 mg/l NAA as the most efficient medium for stevia rooting.

It is known that plantlet produced through stem cutting are unstability, therefore micropropagation of Stevia may overcome many of the limitations associated with conventional method and this can be used for rapid multiplication.
This study aimed to in vitro multiplication of leaf, stem and root explants of two Stevia (*Stevia rebaudiana* Bertoni) genotypes in different tissue culture media for multiplication.

MATERIALS AND METHODS

The present study was conducted in the tissue culture laboratory of the Faculty of Agriculture and Natural Sciences, Recep Tayyip Erdoğan University. Two Stevia (*S. rebaudiana* Bertoni) genotypes originated from Bafra/Turkey and China were used as seed material.

Sterilization

The surface sterilization of Stevia (*S. rebaudiana* Bertoni) seeds washed one as follows: the seeds were first washed 10 min under a flowing tap. Then the seeds were sterilized with alcohol (ethanol 70%) for 2 minutes, then washed three times with distilled water. Further, the same seeds were sterilized with NaOCl (3%) for 5 minutes and washed again with sterilized water up to cleaned from NaOCl. 10 sterilized seeds were transferred then to Magenta caps containing pure MS media. Each magenta cap was wrapped with aluminum folia and incubed in a growth chamber programmed with an 16/8 h light/dark cycle and 25±2 °C temperature (Fatima and Khan, 2010).
The tissue culture media used for the multiplication of stevia seeds are listed in Table 1.

Table 1. Tissue culture media

Pure MS
MS+1 mg/lt BAP + 2 % sugar
MS+1,5 mg/lt BAP + 2 % sugar
pH = 5.7

Explants (root, stem, leaf) were obtained from 15 days old plantlets. 1 cm long explants were transferred to petri dishes (10 explants/petri dish). For regeneration the petri dishes were incubed in a growth chamber programmed with an 16/8 h light/dark cycle and 25±2 °C temperature. Developed shoots were transferred in tissue culture media containing 1 mg/L IAA for rooting. Rooted plantlets were transferred first to sterilized soil and stayed for 15 days in a pre-acclimatization room. After that, they were transferred to outer conditions. Obtained values were calculated using Excel.

RESULTS AND DISCUSSION

The highest shoot regeneration was obtained in both genotypes in stem explants placed in tissue culture media MS+1,5 mg/L BAP. The lowest shoot regeneration was obtained from leaf explants (Table 2, Fig.1) in both genotypes cultiavted on pure MS culture media.

Table 2. Regeneration frequencies and standard deviation values of *Stevia* genotypes obtained from different explants

Plant material	Tissue culture media	Stem explant	Root explant	Leaf explant
Bafra Material	MS	1,66 ± 0,57	2,33 ± 0,5	0,33 ± 0,5
	MS+1 mg/lt BAP	6,0 ± 2,6	4,0 ± 2,6	3,0 ± 1,0
	MS+1,5 mg/lt BAP	11,33 ± 2,08	8,0 ± 2,0	3,0 ± 1,0
Chinese Material	MS	3,33 ± 1,1	0,66 ± 0,1	2,33 ± 1,00
	MS+1 mg/lt BAP	5,0 ± 1,0	7,33 ± 2,08	2,66 ± 0,5
	MS+1,5 mg/lt BAP	24,0 ± 3,6	12,3 ± 2,5	3,33 ± 1,00

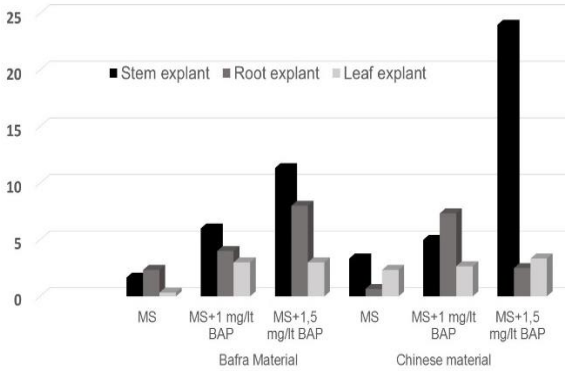

Fig. 1. Regeneration frequencies of Stevia genotypes from different explants

A clear tissue culture media, genotype and explant interaction have been seen in this experiment (Fig. 1). In every genotype obtained plants increased from explant type up to tissue culture media.

In Table 3 and Fig. 2 the number of rooted plants obtained from different explant after transfer to rooting medium were given. The highest number of rooting plants in both genotypes was developed from stem explants (80% in Bafra material and 85,7% in Chinese material). The number of obtained and rooted plants originating from root and leaf explants were remarkably lower in both genotypes (50% and 0% in Bafra material and 62,5 and 25%) in Chinese material.

Table 3. Number of plants obtained from explants transferred to rooting medium

Plant material	Media	Explant	Nr. transferred plants	Nr. of rooted plants	Obtained plants (%)
Bafra Material	MS+1 mg/lt IAA	Stem	20	16	80
		Root	4	2	50
		Leaf	1	0	0
Chinese Material		Stem	28	24	85,7
		Root	8	5	62,5
		Leaf	4	1	25

Taken explants and obtained plants from both genotypes *in vitro* can be seen in Fig. 3 and Fig. 4.

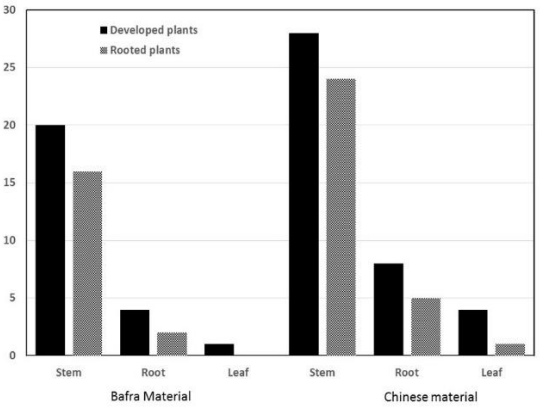

Fig. 2. Number of plants obtained from plantlets originating from different explants transferred to rooting medium

Fig. 3. Explants (3a, 3b) and obtained plants (3c, 3d) from Chinese material

Fig. 4. Explants (4b) and obtained plants (4a, 4c) from Bafra material

Stevia large-scale production is needed for industrial applications. Seeds of *Stevia* show a very low germination percentage (Felippe and Lucas, 1971; Toffler and Orio, 1981), and vegetative propagation through cuttings is limited by the small number of individuals (Sakaguchi and Kan, 1982). Tissue culture is the only rapid process for the mass propagation of *Stevia*.

Biotechnological approaches such as *in vitro* plant tissue culture methods have been applied for the multiplication of stevia all over the world via organogenesis or embryogenesis from different explants for instance axillary shoots, leaves (Ferreira and Handro, 1988), stem tips (Tamura et al., 1984a), nodal segments (Ahmed et al., 2007), suspension cultures (Ferreira and Handro, 1988) and anthers (Flachsland and Mroginski, 1966) and stems (Miyagawa et al., 1984).

The induction of direct shoot regeneration depends on the nature of the plant organ from which the explant was derived and is highly dependent on plant (George, 1993). In our study, the number of obtained and rooted plants in every genotype using different explants was different.

We found that the stevia plant was able to multiplicate using different explants. It can be concluded that the investigated materials should be efficiently multiplicated by using the stem explants.

CONCLUSION

In vitro propagation can be considered an important alternative to conventional propagation and breeding procedures for *S. rebaudiana* which is both an industrially and medicinally important herb. The explants and plant growth regulators levels have significant impact on accelerated micropropagation of *Stevia* to regenerate, genetically true to the type propagules.

The success of *in vitro* culture depends mainly on the growth conditions of the source material, medium composition, culture conditions and on the genotypes of donor plants (Tiwari et al., 2013). İn our case the Chinese genotype showed higher regeneration capacity compared with Bafra material.

Although present results are promising, only two genotypes were used in this study. Therefore to improve this herb's potential as a crop by developing improved varieties with commercially significant yield. there is a need for further improvement, research and development to be carried out.

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