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STUDIES ON SOMATIC EMBRYOGENESIS OF *STEVIA REBAUDIANA* (BERTONI). BY SCANNING ELECTRON MICROSCOPE (SEM)

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ABSTRACT

Development of somatic embryos from nodal and leaf callus of *Stevia rebaudiana* plant has been studied in detailed by Scanning Electron Microscope (SEM). Friable embryogenic callus by single spherical shaped cell aggregates with a diameter of about 20µm diameter is observed. They act as initial cell of somatic embryos or embryo mother cells. The fine structure of somatic embryos was clearly identified during the following sequential developmental stages: single spherical cell, early globular shape with conspicuous suspensor, typical globular shape, elongated shape, early heart shape, typical heart shape, torpedo shape. The high frequency of somatic embryos was found in callus tissues derived from mature leaves under a two step culture method. Low frequency of somatic embryogenesis was found on the leaf edges associated with very small callus formation and also from the callus tissues derived from young leaves under the one step culture method. In the SEM investigation, it is demonstrated that the sequential events leading to *Stevia* somatic embryogenesis arise through one unicellular pathway derived from *Stevia* somatic embryo mother cells.

KEYWORDS: *Stevia rebaudiana*, Tissue culture and Scanning Electron Microscope (SEM).

INTRODUCTION

Stevia rebaudiana Bertonii is a perennial herb belongs to the Asteraceae family. It is a natural sweetener plant known as "Sweet weed", "Sweet Leaf", "Sweet Herbs" and "Honey Leaf", which is estimated to be 300 times sweeter than cane sugar. [1, 4]

The *Stevia* leaves are the significant resource of diterpene glycosides, like rubsoside, steviolbioside, dulcoside, rebaudiosides and stevioside. [9] Amid these compounds, stevioside ranks top in dramatically accelerated use in health concerns related to dental cares, diabetes and obesity. The sweetness is due to stevioside, the most abundant is stevioside. [2] *Stevia* finds its use as a natural sweetener replacing chemical sweeteners and even table sugar. [3] Various studies have found the leafs to contain protein, fibers, carbohydrates, iron, phosphorous, calcium, potassium, sodium, magnesium, zinc, rutin (flavonoid), true vitamin A, vitamin C and an oil which contain 53 other constituents.

The main problem in cultivation of these plants is that they are heterozygous and self-incompatibility leads to low germination percentage and with that vegetative propagation too is limited by the lower number of individuals that can be obtained simultaneously from a single plant. To overcome all these, multiplication and improvement of this medicinal plant though tissue culture may be an alternative for rapid mass propagation of *Stevia*. Somatic embryogenesis and organogenesis have been the common pathways for Clonal propagation of superior medicinal plant species. Somatic embryogenesis enables large numbers of plantlets to be organogenesis. Somatic embryogenesis enables large numbers of plantlets to be produced With in a short span of time.

Somatic embryogenesis has been described for more than hundred plant species [11] but the number of reports of Somatic embryogenesis among members of the Asteraceae is still low.[6]

Previously in many systems Somatic embryogenesis is improved by supplying a source of reduced nitrogen, such as specific amino acids or casein hydrolysate. Somatic embryogenesis in *Stevia* using different concentrations of hormones, sucrose concentrations, addition of amino acid, has been reported but no reports described the ontogenesis of somatic embryogenesis through SEM observations. Callus initiation and growth from *Stevia* leaf explants were previously examined. However developmental sequence of somatic embryos from early stage to differentiation to the final stage has not been fully described in the literature.

In this study, somatic embryo formation will be described at the SEM level especially after transfer to the secondary medium using two step culture method. Somatic embryogenesis usually proceeds in two distinct stages. In the initial stage (embryo initiation), a high

concentration of 2, 4-D is used. In the second stage (embryo production) embryos are produced in a medium with no or very low levels of 2, 4-D.

MATERIAL AND METHODS

Leaves and nodal region of *Stevia rebaudiana* were used as explants sources. The study was kindly provided in Department of Botany, Osmania University. Leaf sections derived from fully matured leaves were inoculated into a primary medium consisting of modified MS medium[7] supplemented with different concentrations of 2, 4-D and 2ip.

The secondary medium was supplemented with a combination of BAP (0.5mg/l) + KN (0.4mg/l). The explants were cultured for 45 days in primary medium and later on whole plant with callus were shifted to the secondary medium. For one step culture method very young leaves (about half size of matured leaves) and fully expanded matured leaves were used. The explants were cultured on modified MS medium supplemented with 0.5 mg/l BAP as the sole source of growth regulator.

For SEM observations, the embryogenic callus at different stages was used for the SEM studies. The callus samples were processed by fixing 4% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 6.8) for about 3 hrs of duration. The samples were washed thoroughly in distilled water 3 times. Then the samples were dehydrated by passing through acetone series from low to high concentrations [20, 40, 60, 80,100% (w/v)] each lasting about 30 minutes of duration. The samples JFC – 1100E/JEOL – 100 CX –BALZAR'S - 4 CD – Ion Sputtering device". After gold coating, the material was observed under" JEOL – JSM – 5200/JEOL – 100CX – Scanning electron microscope" and different stages of somatic embryogenesis were photographed.

RESULTS

A fast growing pale yellow and soft callus was observed at 45 days of culture. After transfer to secondary medium, the growth of this soft callus ceased and the tissue slowly turned brown, and later became almost black in colour. At about 60 days after transfer to secondary medium, a friable yellow callus was very easy to separate into small pieces when BAP + KN were used in combination in secondary medium, the highest efficiency of somatic embryogenesis was observed in BAP(0.5mg/l) + KN(0.4mg/l).

A general surface morphology of a cluster of friable embryogenic callus undergoing the differentiation is seen in fig (1) the callus surface was rough in appearance and yellow in colour. The callus was comprised of two different parts one was a friable yellowish callus and the other a brownish callus. The friable yellowish callus is composed of cells with a spherical shape and the brownish callus consists of elongated cells and a membranous layer covering them. Sometimes the spherical cells are also covered with a membranous layer. The arrangement of both spherical isodiametric cells and an elongated tubular cell is clearly illustrated in the figures. The large nodules are composed of spherical cells as shown in the figure. Under high magnification, the arrangement of isodiametric spherical cells on the surface of a cluster of a friable embryogenic callus can be easily observed. This spherical structure is growing, putative pro embryogenic stage is derived from a single cell of about 15µm in diameter.

One of the fig (2) illustrates that two single cells are of about 20µm in diameter and are interconnected and that a membranous layer is slightly covering both cell surfaces. In the advanced stages of differentiation, spherical cells are sprouting from the basal region of a developed embryo. This differentiation pattern clearly illustrates secondary embryo formation from primary *Stevia* somatic embryos.

Early globular embryos with well developed suspensors have been observed. Fig (3) also shows that a typical globular embryo stage is emerging from the callus surface still interconnected by the suspensor. An early heart shaped embryo with a slight depression on the tip and one slight elongated form of one globular embryo has been observed fig (5). In fig (4) shows the state that typical heart shaped and globular shaped embryos are emerging from a friable embryogenic callus located in the vicinity of well developed embryos. This developmental pattern illustrates embryo formation at the border between developed embryos and friable embryogenic callus fig (6).

The sequential stage of *Stevia* somatic embryo differentiation in the one step method is same as in two step method. However the frequency of embryo formation is less in one step method when compared to two step method and the time period from plant inoculation to regeneration is more in two step method (166 days) when compared to one step method (100 days). Most preferred method is two step method for somatic embryogenesis in plants.

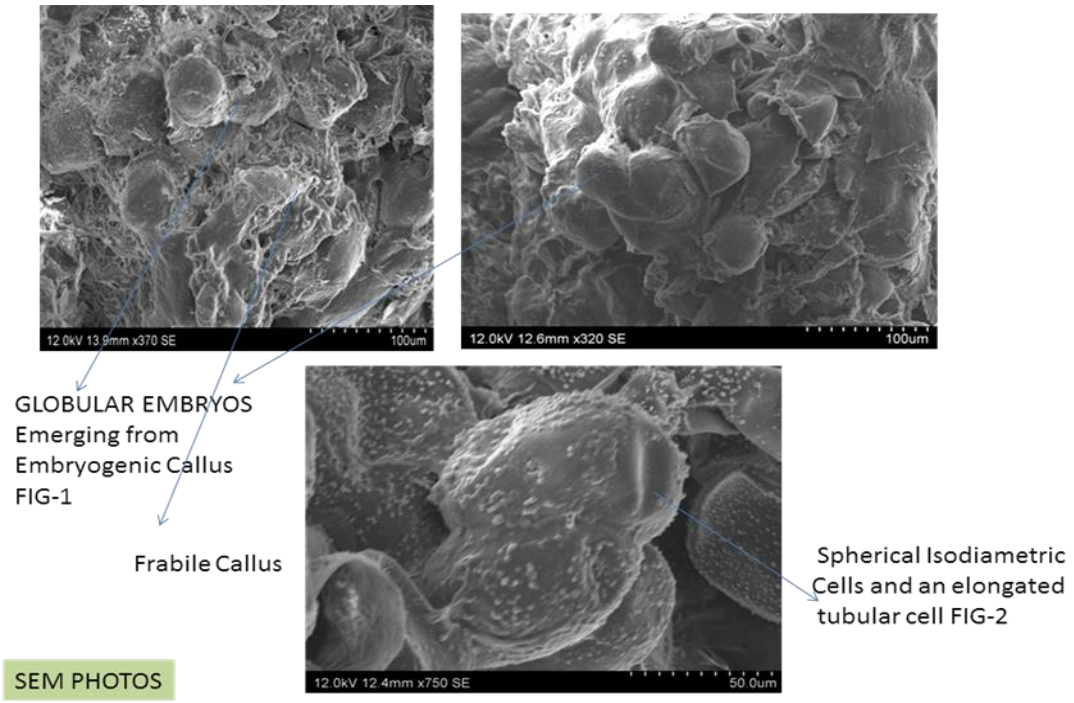
Developmental Pattern of Somatic Embryogenesis

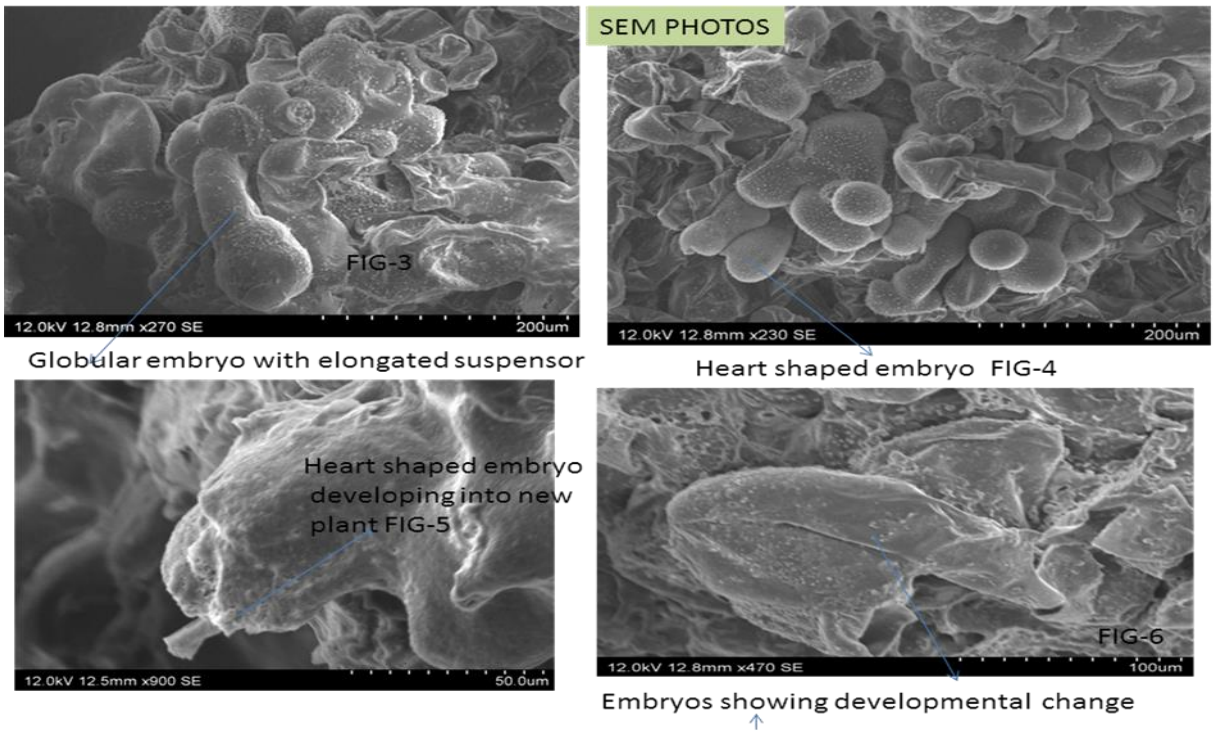
Based on the SEM observation on the somatic embryogenesis in *Stevia* tissue cultured various stages of embryo differentiation in this system can be seen. There are seven distinct stages recognized in the somatic embryogenesis process of *Stevia* tissue culture in this present work cited.

1. Somatic single cell or embryo mother cell
2. Early globular embryo
3. Globular embryo
4. Elongated embryo
5. Early heart embryo
6. Heart embryo
7. Torpedo embryo.

DISCUSSION

Regeneration of somatic embryo genesis has been reported in many dicot plants but there are few papers studying the morphological details by scanning electron microscope especially in perennial plants. A pioneer work on Induction of somatic embryogenesis from leaf explants of *Stevia* was done by Japan in 1933.JOÃO CARLOS BESPALHOK FILHO et al 1993 also published related work showing the effect of increased concentration of sucrose on somatic embryo production. The developmental patterns of somatic embryos were demonstrated by SEM in *Gossypium hirsutum* L. [8, 5] The somatic embryos have a well defined suspensor and an apical indentation of cotyledon representing the initiation of the heart stage. The same features are described in this study with the *Stevia* system. Somatic embryo formation from zygotic embryos has been reported with early cotyledonary stage, and fused embryos in *Cornus florida* L. [12] Also a similar shape of fused embryo was observed with *Stevia* somatic embryogenesis.





In conclusion, the present SEM studies were able to clearly demonstrate that somatic cells from *Stevia* leaf explants could develop into fully differentiated somatic embryos through the characteristic embryological patterns of differentiation. Similar type of SEM investigation has been observed in Coffee plant by Tiemi et al. [10] To further study the structural details of *Stevia* somatic embryos, it will be necessary to make cyto - histological studies on the developmental process of somatic embryos as developed in this paper.

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REFERENCES

1. Chalapathi, M.V. and S. Thimmegowda,. Natural non calorie sweetener *Stevia* (*Stevia rebaudiana* Bertoni): A future crop of India. Crop Research Hisar, 1997; 14(2): 347-350.
2. Kinghorn, A.D., 1992. Food Ingredient Safety Review: *Stevia rebaudiana* Leaves. Herbal Research Foundation, Boulder, CO USA.
3. Kumar, J.K. and V.K. Kaul,. *Stevia rebaudiana* a gift of nature .Indian J.Nat.Prod, 2005; 21: 14-15.
4. Liu, J. and S.F.Y. Li,. Separation and determination of *Stevia* sweetners by capillary electrophorosis and high performance liquid chromatography. J. Liquid Chromatography, 1995; 18(9): 1703-1719.
5. Mat r.a. & trigiano,r.n. Somatic embryogenesis and plant regeneration from leaves of *Dendranthana grandiflora*. Journal of American Society for Horticultural Science, 1991; 116(2): 366-371.
6. Murashigue, t. & skoog, f. A revised medium for rapid growth and biossays with tobacco tissue culIures. Physiologia Plantarum, 1962; 15: 473-497.
7. Shoemaker, R.C., S.E. Christofferson and D.W. Galbraith. Storage protein accumulation patterns in somatic embryos of cotton (*Gossypium hirsutum* L.). Plant Cell Rep, 1987; 6: 12-15.
8. Starrat, A.N., C.W.Kirbyb, R. Pocsa and J.E. Brandle,. Rebaudioside F, a diterpene glycoside from *Stevia rebaudiana*. Phyto cjemistry, 2002; 59: 367-370.
9. Tiemi et al., 1992, Studies on somatic embryogenesis of coffee by SEM. Jpn .J. Crop Sci, 1992; 61(3): 476-486.
10. TERZI M. and LOSCHIAVO, F. Somatic embryogenesis pn: Bhojwani, S.S. Plant Tissue Culture, Applications and limitations; Development in Crop Science. Elsevier, 1990; p. 54-66**.**
11. Trigiano, R.N.M., R.M.Beaty and J.T. Dietrich 1989. Somatic embryogenesis and Plant regenertion in *Cornus florida*. Plant Cell Rep. 8: 270-273.