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# In vitro Micropropagation of *Stevia rebaudiana* Bertoni

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**ABSTRACT: *Stevia rebaudiana* Bertoni is a herbaceous perennial plant, belonging to family Asteraceae. *Stevia* leaf extract exhibits a high degree of antioxidant activity and has been reported to inhibit human cancer cell growth. Plant tissue culture is a suitable approach for micro propagation and production of valuable secondary metabolites of plants. The aim of present study was to develop a suitable protocol for micro propagation of *Stevia rebaudiana* Bertoni. The experiment was conducted as a completely randomized design in a factorial arrangement (2 media × 5 hormone concentrations = 10 treatments) with three replicates. Analysis of variance showed that the effect of activated carbon was significant ($p < 0.05$; or $< 0.01$) for all traits except the number of shoots. The results also showed significant differences among hormone concentrations in all traits except the mean number of node and axillary shoots. The significance of media × hormone interaction suggests that the effects of hormone concentration are influenced by media for all traits except number of node.**

**Keywords:** *Stevia rebaudiana*, micropropagation, activated carbon, hormone

## INTRODUCTION

*Stevia rebaudiana* Bertoni is a perennial small shrub that belongs to the family Compositae. This medicinal plant has herbaceous growth habit and growing of plants require mild temperature and relative humidity of about 80% (Singh and Rao, 2005; Soliman *et al.*, 2014). The leaves of stevia are the source of diterpene glycosides, stevioside and rebaudioside. Because of these components, this herb is a natural sweetener plant, estimated to be 300 times sweeter than sugar cane (Soejarto *et al.,* 1982; Yoshida, 1986). Stevioside is regenerated as a valuable natural sweetening agent due to its relatively good taste and chemical stability. It is of special interest to diabetic persons with hyperglycemia and the diet conscious (Arpita *et al.,* 2011; Toyoda *et al.*,1997). The seeds of this plant show very low(less than 10%), germination rates(Toffler and Orio 1981). In addition, due to self incompatibility which results in sterile seeds, the production of homogeneous populations is not possible. (Jagatheeswari and Ranganathan 2012; Hossain *et al.* 2008).

Plant tissue culture as a technique of growing explants isolated from the mother plant, includes different methods used for micro propagation, callus induction and production of valuable secondary metabolites of plants (Vyas and Dixit, 1999). Through plant tissue culture, the totipotent cells of plant can be used for the in vitro regeneration of plant, so this technique is a suitable approach to prepare sufficient amount of plant materials within a short span of time in large scale and enhance the natural levels of in vitro production of valuable compounds (Sung 2006; Pande and Gupta, 2013) .

The use of micropropagation has been reported for a number of medicinal plants (Naik, 1998). There are some reports that clearly support the possibility of propagating *S. rebaudiana* by tissue culture techniques (Razak *et al.*, 2014; Uddin *et al.*, 2006; Pande and Gupta, 2013). Uddin *et al.* (2006) established in vitro propagation from different explants of *S. rebaudiana* Bertoni using MS medium supplemented with certain plant growth regulators (Uddin *et al.*, 2006). There are various reports of in vitro propagation of *Stevia* using different explants (Akita *et al.* 1994; Salim *et al.* 2006; Uddin *et al.* 2006). The present study was undertaken to find out the suitable medium and the best combination of plant growth regulators for n micro propagation of *Stevia rebaudiana* Bertoni.

## MATERIALS AND METHODS

The experiments of the present study were conducted in the Tissue Culture and Genomics Laboratory, Kermanshah branch, Islamic Azad University, Kermanshah, Iran.

*A. Plant Material and Surface Sterilization*

The nodal segments comprised the explants which were collected from four months old plants. The explants were cut into small pieces (about 2 cm long) and then were put under running tap water. The explants were surface sterilized by dipping in 70% Ethanol for 30 Sec, then dipping in 2.5% (v/v) sodium hypochlorite solution for 4 min with constant shaking, followed by three rinses with sterile distilled water.

*B. Media and culture conditions*

MS (Murashige and Skoog, 1962) medium with 30 g $L^{-1}$ of sucrose was used as the basal medium. The medium was solidified using 7 g $L^{-1}$ of agar. The medium was modified by adding of 2 g $L^{-1}$ activated carbon(AC). MS medium was supplemented with various concentrations of plant regulators (Table 1). The pH of the medium was adjusted to 5.6-5.8 before adding agar and activated carbon, then the culture medium was autoclaved at 121°C for 20 min. All the cultures were maintained in growth room at 25±2°C, 16 h light/8 h dark photoperiod.

*C. Experimental design and Statistical analysis*

The experiment was conducted as a completely randomized design in a factorial arrangement (2 media × 5 hormone concentrations = 10 treatments) with three replicates (each bottle as a replicate) and five explants per plot. Shoot length, the mean number of node, axillary shoot, leaf per shoot, alive plant per bottle and the weight of shoot and root were recorded 35 days after planting. The analysis of variance (ANOVA) was carried out using MSTAT-C software. The purpose of ANOVA was to find out significant differences between treatments in studied traits and Duncan's multiple range test was used for mean separation.

**Table1: The list of medium and hormons used in this study.**

| Media | Hormone | Treatment |
|---|---|---|
| Non activate carbon | Ms full | 1 |
| | Ms full + BAP (0.5) mg/l | 2 |
| | Ms full + 2,4-D (0.5)mg/l | 3 |
| | Ms 1/2 | 4 |
| | Ms full + NAA (0.1)mg/l | 5 |
| Activate carbon | Ms full | 6 |
| | Ms full + BAP (0.5) mg/l | 7 |
| | Ms full + 2,4-D (0.5)mg/l | 8 |
| | Ms 1/2 | 9 |
| | Ms full + NAA (0.1)mg/l | 10 |

## RESULTS AND DISCUSSION

Analysis of variance for the factorial experiment showed that the effect of activated carbon was significant ($p < 0.05$; or $< 0.01$) for all traits except the number of shoots. The results also showed significant differences among hormone concentrations in all traits except the mean number of node and axillary shoots. The significance of media × hormone interaction suggests that the effects of hormone concentration are influenced by media for all traits except number of node (Table 2). The maximum number of alive plants (3.22) was found in the MS medium supplemented with 0.5 mg $L^{-1}$ BAP without activated carbon after five weeks of cultivation. Maximum number of leaf per shoots were achieved on MS medium supplemented with 0.5 mg $L^{-1}$ 2,4-D. The highest shoot length was observed in the free hormone MS medium without activated carbon. According to Das *et al.* (2011) the highest shoot length of stevia was achieved when Kinetin was present in the medium.

**Table 2: Analysis of variance table for a completely randomized design in a factorial arrangement.**

| S.O.V | df | Mean of squares | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **Shoot length** | **Shoot number** | **Node number** | **Leaf per shoot** | **Alive plant** | **Shoot fresh weight** | **Root fresh weight** | **Shoot dry weight** | **Root dry weight** |
| Medium | 1 | 18.63** | 1.93 | 13.7* | 176.3* | 4.65** | 0.1** | 0.024** | 0.001** | 0.00007** |
| Hormone | 4 | 0.93* | 0.7 | 3.98 | 37.7* | 2.1** | 0.018** | 0.014** | 0.00009* | 0.00003** |
| Medium × Hormone | 4 | 0.84* | 3.22* | 5.9 | 54.7* | 2.2** | 0.021** | 0.015** | 0.0001** | 0.00003** |
| Error | 20 | 0.23 | 0.75 | 2.23 | 22.5 | 0.27 | 0.002 | 0.001 | 0.00002 | 0.000001 |

*, ** means significant at alpha= 0.05 , 0.01 respectively

**Fig. 1.** Development of more number of multiple shoot after 5 weeks.

The hormonal treatments did not differ significantly in the mean number of axillary shoots. According to Razak *et al.* (2014), the maximum number of shoots were observed on MS medium supplemented with 0.5 mg $L^{-1}$ BAP and 0.25 mg $L^{-1}$ Kinetin (7.82 ± 0.7) after four weeks of cultivation. Tadhani *et al.* (2006), also reported that the highest number of shoots were achieved on MS medium supplemented with 0.6 mg $L^{-1}$ of BAP. According to Sivaram and Mukudan (2003), The combination of low concentration of BAP and low concentration of Kinetin induced high number of multiple shoots and higher concentration of BAP resulted decreasing multiple shoots formation of stevia. Anbaznagan *et al.* (2010), reported that the combination of BAP and Kinetin work well for booth shoot proliferation and their elongation from the nodal explants.

The maximum shoot fresh weight (0.38gr) and shoot dry weight (0.05gr) were obtained in the MS medium supplemented with 0.5 mg $L^{-1}$ of BAP. However, Maximum root fresh weight (0.23 g) and root dry weight (0.02 gr) were observed in the free hormone MS medium. According to Hwang (2006), the use of 1.0 mg $L^{-1}$ IBA in the MS medium induce maximum numbers of roots. Tadhani *et al.* (2006) also observed the highest rate of root induction in MS medium supplemented with 1.0 mg/L IBA.

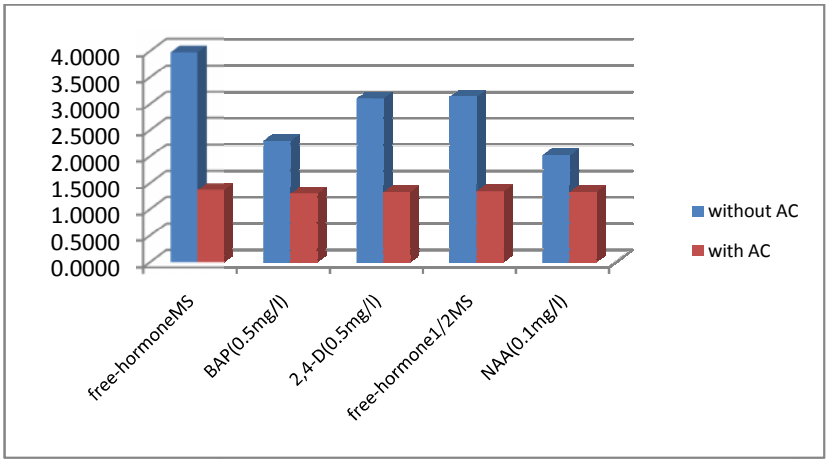


**Fig. 2.** Shoot length of stevia plantlets on different media.

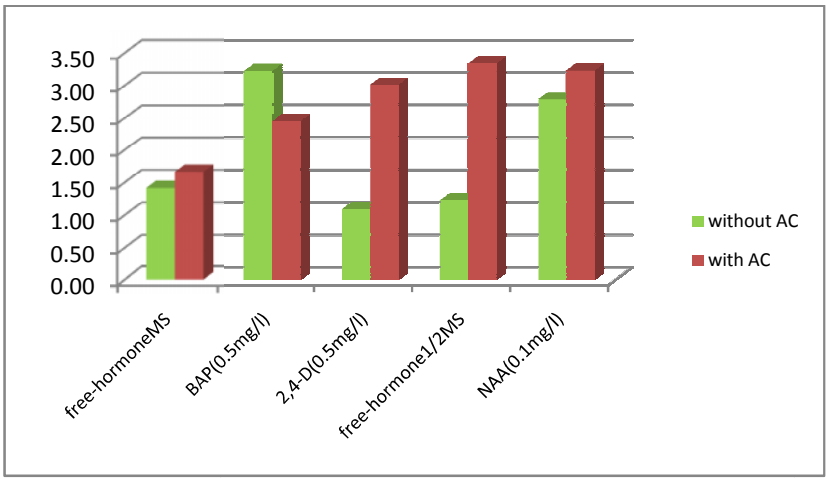


**Fig. 3.** Number of Alive plants on different media.

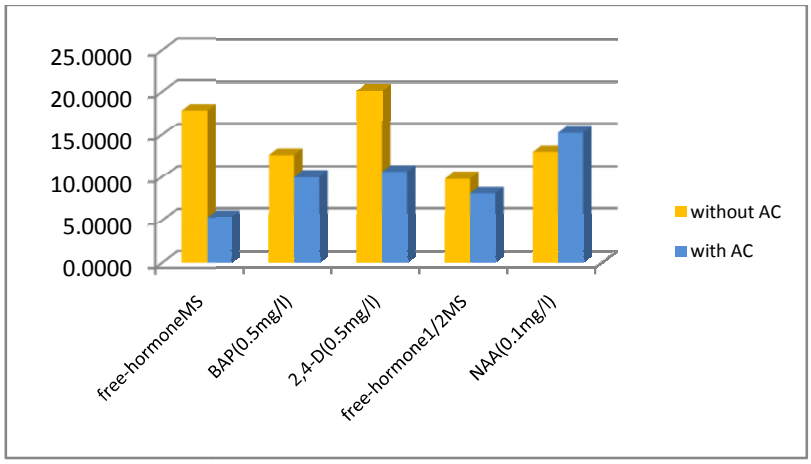


**Fig. 4.** Number of leaf per shoot on different media.

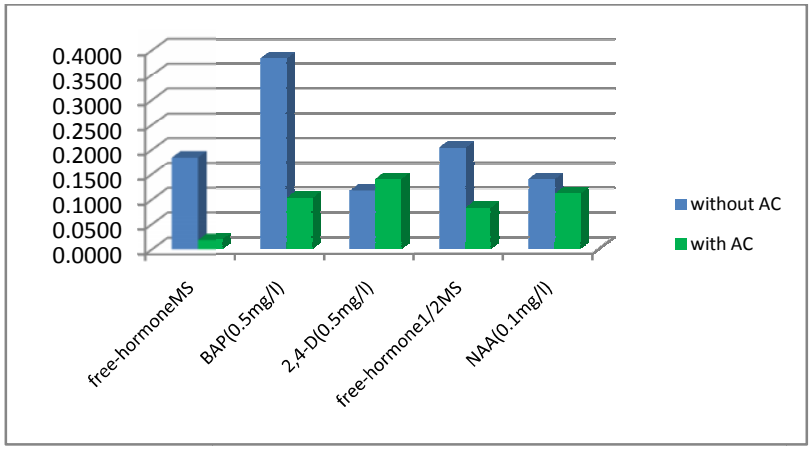


**Fig. 5.** Shoot fresh weight of stevia plantlets on different media.

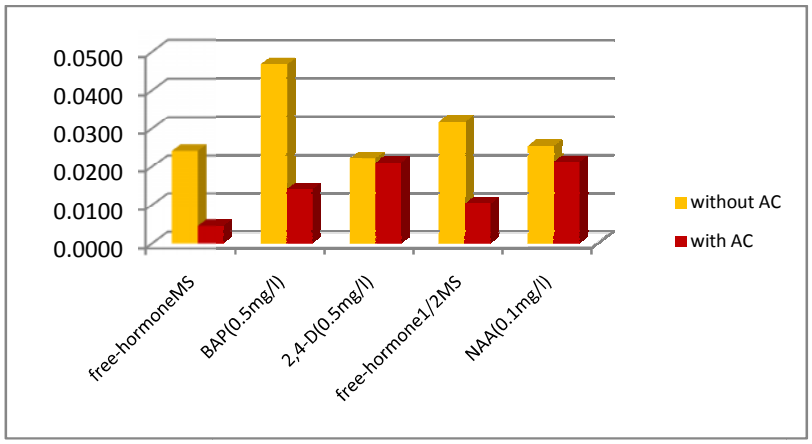


**Fig. 6.** Shoot dry weight of stevia plantlets on different media.

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