IN-VITRO PROPAGATION OF STEVIA REBAUDIANA (BERTONI) BERTONI (ASTERACEAE)

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Abstract - Stevia rebaudiana (Bertoni) Bertoni is a medicinal plant native to South America, which produces diterpene steviol glycosides which are low calorie sweeteners, about 300 times sweeter than sucrose. Even though, this plant has an immense potential to be used in Sri Lanka, it has not been introduced yet due to various reasons. The main objective of the present study is to find out an optimum protocol for in-vitro propagation of Stevia rebaudiana. Explants were collected from authenticated mother plants maintained in the green house. Nodal explants were surface sterilised using different concentrations of Clorox^{R}. Culture establishment and shoot multiplication were performed in Murashige and Skoog medium (MS) supplemented with different concentrations and combinations of BA and IBA. In vitro rooting of shoots were tested using different concentrations and combinations of IBA and charcoal. Data were analysed using ANOVA in SAS statistical package. Results exhibited that the optimum surface sterilization method for Stevia rebaudiana is 15% clorox for 15 minutes. MS medium supplemented with 0.5mg/L IBA and 2mg/L BA, exhibited the best shoot proliferation rate. Itcan be sub cultured up to four sub cultures without any significant decline in the number of shoots produced per plant. The optimum rooting was observed in MS medium supplemented with 0.1% charcoal and 1mg/L IBA. Rooted plantlets were successfully allimatied with 85% success rate.

Index Terms - In-vitro propagation, Stevia rebaudiana, Nodal explant, MS medium, Large scale production

I. INTRODUCTION

Due to overuse of sweeteners and sedentary life styles of people, the incidence of obesity and diabetic conditions has been increased. Because of the high health risk of sugar and chemical sweeteners, the interest is now being focused on natural non-caloric sweeteners globally. The scenario of sweeteners has changed towards a sweeter change in the recent past with the introduction of stevia, a non-caloric, natural sweetener. Stevia rebaudiana (Bertoni) Bertoni (Asteraceae) is a perennial herb, which is cultivated for its sweet leaves. Stevia leaves are 250 to 300 times sweeter than the sucrose due to presence of natural, non caloric sweeter compounds steviol glycosides of its leaves [1],[2],[3],[4]. Since the use of stevia does not affect glucose levels in the human body, it is being used for the treatment of diabetes. Therefore, there is an increasing demand for stevia due to ever increasing diabetes disorder among the population [5]. Conventionally, Stevia can be propagated through seeds; but it is not successful due to several physiological reasons including low seed viability, less dormancy etc. [7][8]. Moreover, vegetative propagation of stevia through rooting of stem cuttings are also affected by several factors such as requirement of large number of cuttings, low rate rooting, and low success in direct planting in the field [9]. Meanwhile, plant tissue culture technique has been identified as a rapid propagation method for many commercially important plant species [10]. Therefore, the aim of the present study is to investigate a successful in vitro protocol for mass propagation of S. rebaudiana for commercial cultivation in Sri Lanka.

II. METHODOLOGY

2.1. Collection of explants and sterilization

Nodal explants were obtained from authenticated, healthy mother plants maintained at institutional greenhouse. Collected nodal explants were washed with liquid detergent (Teepol) solution and subsequently with running tap water for 45 minutes. Then they were dipped in fungicide solution for 30 minutes. Then the explants were disinfected with 10% Clorox solution for 15 minutes and 15% Clorox solution for 10 minutes followed by rinsing them for five times with sterilized distilled water.

2.2. Shoot multiplication

Nodal explants collected from the mother plants were cultured on Murashige and Skoog, 1962 basal medium (MS medium) augmented with 3 % sucrose and solidified with 8g/L agar, fortified with different concentrations of BAP (0-–3.0 mg/L) and IBA (0.–2.0 mg/L) for shoot multiplication. pH was adjusted to 5.6 using a pH meter (330i/SET, Germany). Growth data were recorded after 6 weeks of culture establishment.

2.3. Rooting of elongated shoots and acclimatization

The elongated shoots (>4.0 cm height) were transferred to MS medium supplemented with different concentrations of IBA (1–2 mg/L) and activated charcoal (0.1-0.2 mg/L) for root induction.

After 4 weeks, plantlets with well-developed roots were uprooted and gently washed under running tap water to remove adhering medium. Subsequently, they were established in pots containing sterile sand: coir dust: compost in 1:1:1 medium and they were initially maintained in the controlled environment for two weeks and subsequently transferred to the greenhouse. After twenty days, the plantlets were successfully established in the field.

2.4. Shoot multiplication

In order to achieve multiple shoots, the shoots were subcultured in the medium with 0.5mg/L IBA & 2mg/LBA combination and maintained for 6 weeks This process was repeated for another four subcultures (each 6 weeks) to examine the effect of subculture on production of large scale shoot buds. Data on number of shoots per each subculture was recorded.

2.5. Statistical analysis

Experiments were set up in a completely randomized block design (CRD). Mean values were compared by using General Linear Model of ANOVA followed by Tukey Multiple Range Test Using SAS (SAS Institute, 1999). The p values less than 0.05 were adopted as statistically significant.

III. RESULTS AND DISCUSSION

3.1 Establishment of suitable surface sterilization procedure

Selection of a suitable disinfectant and its optimum concentration for sterilization of explants is a key factor of in vitro propagation. Results of the current study demonstrated that, out of two treatments tested, 15% sodium hypochlorite (NaOCl) for 15 minutes is the optimum level of disinfectant for surface sterilization of shoots and nodal explants of Stevia. Results of the current study are agreement with Kittisak Chotikadachanarong and Srisuluk Dheeranupattana [11], who successfully sterilized stevia explants by using NaOCl.

3.2 Shoot multiplication

Results clearly demonstrated that, irrespective of the medium composition and hormone levels, all treatments were effective for shoot induction, producing of more than 2 shoots per explant after 6 weeks of culture establishment. The best shoot multiplication was observed in MS medium supplemented with 0.5mg/L IBA and 2 mg/L BA. Also it was observed that, when IBA concentration was increased, callusing was also increased and subsequently reduced the number of shoots in the cultures. Moreover, it was observed that, there was a positive correlation with BA level and number of shoots up to 2 mg/L of BA, and numbers of shoots were decreased when BA concentration increased up to 3 mg/L (Table 1). Our results are in agreement with Buhara Yücesan, (2016), who compared the effect of different auxins and cytokinins levels for the shoot multiplication and observed that 2mg/L of BA is the best hormone concentration for the shoot multiplication of S. rebaudiana.

3.3 Induction of multiple shoots

The nodal explants derived invitro regenerated shoots were cultured on MS medium supplemented with 2.0 mg/L BA and 0.5mg/L IBA for 6 weeks and repeated for subsequent 5 subcultures. Results revealed that there was no significant difference of average shoot number up to 4 subcultures. However, in the fifth subculture there was significant reduction of shoot number.

Table 1: Effect of different concentrations of plant growth regulator on shoot bud induction from nodal explants of Stevia rebaudiana (Means with different letters are significantly different at p= 0.05)

Hormone levels		No of shoots
IBA (mg/L)	BA (mg/L)	
-	-	2.33 ± 0.67^{c}
-	1	2.875 ± 0.59^{c}
-	2	3.375 ± 0.48^{ab}
-	3	3.22 ± 0.41^{b}
0.5	-	3 ± 1.26^{b}
0.5	1	3.4 ± 0.48^{a}
0.5	2	3.857 ± 0.63^{a}
0.5	3	3.285 ± 0.43^{a}
1	-	2.11 ± 0.73^{b}
1	1	2.33 ± 0.67^{c}
1	2	2.44 ± 0.68^{c}
1	3	2.6 ± 0.48^{b}

3.4 Rooting of shoots and acclimatization

As demonstrated in Table 2, rooting of in vitro propagated shoots revealed that irrespective of treatments, all shoots produced roots in MS medium. However, the best performance by means of rooting percentage, number of roots per shoot and root length was observed in MS medium supplemented with 0.1% charcoal and 1mg/L IBA (95 ± 0.26^{a}) followed 0.1% charcoal and 2mg/L IBA ($90\pm0.48^{a\,b}$). However, increased charcoal treatments exhibited negative correlation with rooting percentage. Our results are in agreement with Abd El-Motaleb[12] who observed higher percentage of rooting and root performance of S. rebaudiana at lower concentrations of IBA. Rooted shoots were field established with 85% success.

Table 2. Table 3: Effect of different concentrations of IBA and charcoal on in vitro rooting of elongated shoots of Stevia rebaudiana

Hormone levels		Rooting (%)	Root number	Root length (cm)
Charcoal (%)	IBA (mg/L)			
-	-	80 ± 0.67^{c}	3.62 ± 1.79^{de}	0.3 ± 0.15^{c}
-	1	85 ± 0.59^{b}	7.75 ± 1.68^{b}	0.38 ± 0.28^{bc}
-	2	80 ± 0.48^{c}	5.67 ± 2.49^{cd}	0.44 ± 0.32^{bc}
0.1	-	80 ± 0.41^{c}	7.143 ± 2.81^{bc}	1.57 ± 0.86^{a}
0.1	1	95 ± 0.26^{a}	11.167 ± 1.45^{a}	1.72 ± 0.408^{a}
0.1	2	90 ± 0.48^{ab}	8.167 ± 1.23^{b}	0.92 ± 0.701^{b}
0.2	-	60 ± 0.63^{d}	1.71 ± 0.88^{e}	0.52 ± 0.804^{bc}
0.2	1	45 ± 0.43^{e}	2.75 ± 0.43^{e}	0.77 ± 0.58^{b}
0.2	2	40 ± 0.73^{e}	1.5 ± 0.86^{e}	0.75 ± 1.01^{b}

CONCLUSION

Stevia rebaudiana can be successfully propagated through in vitro propagation system by using stem or shoot cuttings in MS medium supplemented with different concentrations of BA and IBA. The best hormone combination for shoot multiplication and rooting were was 0.5mg/L IBA & 2 mg/LBA and 0.1% charcoal and 1mg/L IBA respectively. In vitro propagated shoots can be maintained up to 4 subcultures without affecting the growth. In vitro propagated shoots can be acclimatized more than 80% success

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