EFFECT OF NITROGEN SOURCES ON ROOTING OF IN VITRO CULTURE OF STEVIA REBAUDIANA (BERTONI)

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ABSTRACT

_Stevia rebaudiana_ Bert. belongs to family Asteraceae. It is a natural sweetener with zero calorie contributing to various medicinal properties like anti-diabetic, anti-hypertensive, anti-microbial, anti-oxidant, anti-aging and anti-cancerous etc. Large scale production is required to meet the increasing demand for the natural sweeteners. Keeping all above mentioned points in view the present study was undertaken to investigate the effect of nitrogen on the rooting of _Stevia rebaudiana_ to develop healthy rooting system for balanced plant physiology. The multiplied shoots were cultured on MS medium fortified with BAP (5.0 mg/l) along with Kinetin (1.0 mg/l). Effect of plant growth regulator IAA (1.0 mg/l) and α-NAA (1.0 mg/l) each in combination along with and without activated charcoal on induction and multiplication of roots on MS medium + NH₄NO₃ (14 N/l) + KNO₃ (400 N/l) was recorded at the interval of two weeks. Results showed that less concentration of NH₄NO₃ (14 N/l) is required as compared with KNO₃ (400 N/l) for better induction and multiplication of shoot and root. Although presence of nitrogen sources improves both shooting and rooting in stevia but the presence of plant growth regulators is must. The present study also advocates the addition of activated charcoal for healthy root development for both their number and length. Survival of 85% was achieved when rooted plantlets were acclimatized in 2 FYM: 1 Perlite: 1Garden Soil.

KEYWORDS: _Stevia rebaudiana_, Micropropagation, Nitrogen, Farmyard Manure

INTRODUCTION

Due to increased incidences of diabetes and obesity in all over the world, and growing concern over the safety of chemical sweeteners, there is an urgent need of natural, non-caloric sweeteners with acceptable taste and health properties. The solution to above mentioned problematic area can be deduced with the herbal drugs extracted from plants.

One of such plants is _Stevia rebaudiana_ Bert. (2n=22) that belongs to Asteraceae family, is a perennial shrub and one of the most valuable tropical medicinal plants. It grows nearly 65 cm, with sessile, oppositely arranged lanceolate to oblanceolate leaves and had serration in middle. The first report of its commercial cultivation in world was from Paraguay in 1964 (Katayama _et al._, 1976; Lewis, 1992). It is also cultivated in countries like Brazil, Korea, Mexico, United States, Indonesia, Tanzania and Canada (Brandle and Rosa, 1992; Fors, 1995). In India, the plant was introduced at the University of Agricultural Sciences, Bangalore during the late 1990s, and studies on its adaptability were initiated and two different accessions were introduced in Palampur for domestic as well as commercial cultivation (Megeji _et al._, 2005). Steviosides, glycosides A and dulcosides are main secondary metabolites of this plant, are sweet crystalline diterpenes and of commercial importance. These are extracted from the leaves of the plant.

These secondary metabolites (Steviosides, glycosides A, dulcosides) are used as functional component in the food industries (Savita _et al._, 2004). Its extracts were 152 times sweeter than 3 per cent sucrose and 97 times sweeter than 10 per
In addition to its zero-caloric sweetening properties, it has many therapeutic values: such as anti-hyperglycaemic, anticancerous (Jeppesen et al., 2002; Jeppesen et al., 2003) and anti-hypersensitive (Chen et al., 2006; Jeppesen et al., 2002), contraceptive properties (Melis, 1999), anti-microbial (Pereda-Miranda and Hernández-Carlos, 2002) and prevents dental caries (Fujita and Edahiro, 1979) etc. Products like stevia sweetener will increasingly be in demand due to consumer interest in natural products. Such demand will need to be supported by large scale production of this plant. But its cultivation is restricted due to self-incompatibility of its flowers and poor seed germinating rate (Savita et al., 2004). The chances of vegetative breeding are also restricted due to high frequency of infection and lower number of individuals (Sakaguchi and Kan, 1982). So the advent of plant tissue culture provides answers to its cultivation related problems. Most commonly used basal medium for micropropagation of *Stevia rebaudiana* is MS (Murashige and Skoog, 1962) in which inorganic nitrogen sources of NH$_4^+$-N and NO$_3^-$-N and NH$_4^+$:NO$_3^-$ = 1:2 are used. As healthy root system of a plant defines the overall growth of plant because all nutrients, water is absorbed through it which are required for balanced plant physiology so in present investigation we attempted to improve the rooting system of *Stevia rebaudiana* by varying the concentration of nitrogen sources along with the best observed concentration of plant growth regulators in modified medium.

**MATERIALS AND METHODS**

**Plant Materials**

The explants (multiplied shoots) were collected from *in vitro* multiplied *Stevia rebaudiana* shoots at the Plant Tissue Culture Laboratory, NIMS University, Jaipur. These shoots were maintained aseptically at basal MS medium supplemented with various concentrations and combinations of different auxins α-NAA (1.0 and 2.0 mg/l) and IAA (0.5 - 5.0 mg/l) and cytokinines, BAP (0.5-5.0 mg/l), kinetin (0.3-5.0 mg/l) alone and in combination BAP (3.0-5.0 mg/l), kinetin (1.0-3.0 mg/l) was used. The multiplied shoots were cut into small pieces with (2-3 nodes) and then were inoculated aseptically on MS medium with modified NH$_4$ and NO$_3$ nitrogen, Kinetin (1.0 mg/l) and BAP (5.0 mg/l) for induction of shoots and same media was used for multiplication of shoots on aseptic conditions.

**Rooting Medium**

Elongated shoots (2-3 cm long) were excised and transferred on MS medium + NH$_4$NO$_3$ (14-56 N/l) + KNO$_3$ (100-400 N/l) along with (0.5 g/l) and without activated charcoal and the media was also supplemented with different concentrations of IAA, α- NAA for induction and proliferation of healthy roots. Data was recorded for six weeks at the interval of two weeks.

**Acclimatization and Transfer of Plantlets to Soil**

Plantlets with well-developed roots were transferred to mist house for hardening which contained autoclaved garden soil, farmyard manure and perlite. Acclimatization was standardized for its time period, relative humidity and temperature conditions before the plants were transplanted in to the soil in field condition.

**Statistical Analysis**

Experiments were set up in a Randomized Block Design (RBD) and each experiment was done with 10 replicates and repeated three times.

**RESULTS AND DISCUSSIONS**

Nitrogen sources NH$_4$NO$_3$ (14-56 N/l) and KNO$_3$ (100-400 N/l) along with Kinetin (1.0 mg/l) and BAP (5.0 mg/l) were observed for their impact on the initiation and multiplication of shoots on MS media (Graph 1). In initial week
maximum number of shoots (6.60 ± 0.67) were observed with concentration 14 N/l, 400 N/l and maximum length (1.90 ± 1.66cm) of shoot was observed with 14 N/l, 100 N/l of NH$_4$NO$_3$ and KNO$_3$, respectively. In second week maximum number of shoots (15.6 ± 0.67) was recorded with concentrations 14 N/l, 400 N/l and maximum length (2.80 ± 2.01cm) of shoot was observed with concentrations with same concentration of NH$_4$NO$_3$ and KNO$_3$. The textures of shoot were healthy, green as compared to other combinations. In fourth week maximum number of shoots (25.0 ± 18.4) and maximum length (4.00 ± 0.44cm) of shoot was observed with 14 N/l, 400 N/l of NH$_4$NO$_3$ and KNO$_3$, respectively. In sixth week maximum number of shoots (33.4 ± 0.55) and maximum length (5.12 ± 0.83cm) of shoot was observed with 14 N/l, 400 N/l of NH$_4$NO$_3$ and KNO$_3$, respectively and shoots were green in colour. The use of kinetin and BAP for initiation and multiplication in MS media was in concordance with (Ahmed et al., 2007; Rafiq et al., 2007).

The plant growth regulator IAA (1.0 mg/l) and α-NAA (1.0 mg/l) each in combination without activated charcoal on induction of root on MS medium with nitrogen was observed (Graph 2). In initial week maximum number of roots (5.4 ± 1.14) were recorded with concentration 1.0 mg/l, 1.0 mg/l and maximum length (1.68 ± 0.30 cm) of root was observed with 0.5 mg/l, 0.5 mg/l of IAA and α- NAA. In second week maximum number of roots (12.6 ± 1.14) were recorded with concentration 1.0 mg/l, 1.0 mg/l and maximum length (2.60 ± 0.34cm) of root was observed with 0.5 mg/l, 0.5 mg/l of IAA and α- NAA respectively. In fourth week maximum number of roots (18.8 ± 1.48) recorded were with concentration 1.0 mg/l, 1.0 mg/l and maximum length (3.28 ± 0.26 cm) of root was observed with 0.5 mg/l, 0.5 mg/l of IAA and α- NAA. In sixth week maximum number of roots (28.0 ± 1.23) were recorded with concentration 1.0 mg/l, 1.0 mg/l and maximum length (3.90 ± 0.25cm) of root was recorded with 0.5 mg/l, 1.0 mg/l of IAA and α- NAA. The morphological observation of multiple shoots were green and of large size. The effect of plant growth regulators such as NAA along with varying concentrations of nitrogen on rooting was also having its similarity with (Wu et al., 2005).

Effect of plant growth regulator IAA (1.0 mg/l) and α-NAA (1.0 mg/l) each in combination along with activated charcoal on induction and multiplication of roots on MS medium + NH$_4$NO$_3$ (14 N/l) + KNO$_3$ (400 N/l) was recorded at the interval of two weeks (Graph 3). In initial week maximum number of roots (4.4 ± 1.14) were recorded with concentration 1.0 mg/l, 1.0 mg/l and maximum length (1.68 ± 0.30 cm) of root was observed with 0.5 mg/l, 0.5 mg/l of IAA and α- NAA respectively. In second week maximum number of shoots (13.6 ± 1.14) and maximum length (2.88 ± 0.29 cm) of shoot was observed with 1.0 mg/l, 1.0 mg/l of IAA and α- NAA. In fourth week maximum number of roots (22.8 ± 1.48) and maximum length (3.78 ± 0.19 cm) of root was observed with 1.0 mg/l, 1.0 mg/l of IAA and α- NAA. In sixth week maximum number of roots (33.0 ± 1.23) were recorded with concentration 1.0 mg/l, 1.0 mg/l and maximum length (4.11 ± 0.19cm) of shoot was observed with 0.0 mg/l, 0.5 mg/l of IAA and α- NAA. The phenotypic variation of plantlets showed dark green, healthy shoots and roots. Both the parameters showed better results in media with activated charcoal as compared with media deficient in activated charcoal. The use activated charcoal and auxins to improve rooting was also supported by the results of Sairkar et al. (2009) as there was significant increase in percentage of rooting in media containing activated charcoal when compared with media lacking the earlier. The use of auxins in medium for rooting was also supported by the results of Arya et al. (2012). Their results of study showed that no rooting occurred without plant growth regulators and presence of auxins increased the percentage of rooting in multiplied shoots.

The permutation of nitrogen on the formation of roots on MS medium along with IAA (1.0 mg/l) and α- NAA was recorded (Graph 4). In initial week maximum number of roots (5.0 ± 0.81) was observed with 42 N/l, 300 N/l and maximum length (1.5 ± 0.47 cm) of root was observed with 14 N/l, 400 N/l of NH$_4$NO$_3$ and KNO$_3$. In second week maximum number of roots (15.2 ± 1.50) and maximum length (3.22 ± 0.26 cm) of root was observed with 14 N/l, 400 N/l of NH$_4$NO$_3$ and KNO$_3$. In fourth week increased number of roots (27.5 ± 1.30) and increased length (4.1 ± 0.19 cm) of root
was observed with 14 N/l, 400 N/l of \( \text{NH}_4\text{NO}_3 \) and \( \text{KNO}_3 \). In sixth week maximum number of roots (31.2 ± 0.88) was recorded with concentrations 28 N/l, 300 N/l and increased length (4.46 ± 0.23cm) of root was recorded with 14 N/l, 400 N/l of \( \text{NH}_4\text{NO}_3 \) and \( \text{KNO}_3 \). The use of nitrogen combinations to develop healthy root system was also advocated that root formation was much better in media devoid of \( \text{NH}_4\text{NO}_3 \) (Sriskandarajahi and Skirvin, 1991; Villamor, 2010). Survival rate of 85% was achieved when rooted plantlets were acclimatized in 2 FYM: 1 Perlite: 1 Garden Soil. No variance was observed between the plantlets which lead to healthy plants production.

CONCLUSIONS

The present study gives the importance of nitrogen sources present in media for the development of healthy root system. Our results suggests that less concentration of \( \text{NH}_4\text{NO}_3 \) (14 N/l) is required as compared with \( \text{KNO}_3 \) (400 N/l) for better induction and multiplication of shoot and root. Although presence of nitrogen sources improves both shooting and rooting in Stevia but the presence of plant growth regulators is must. The present study also advocates the addition of activated charcoal for healthy root development for both their number and length. This study verifies role of nitrogen sources for better results in rooting and shooting system still further research is required in this regard. So present study can be taken as base for further research in the area of plant tissue culture of *Stevia rebaudiana* as well as for other medicinal plants.

REFERENCES


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**APPENDICES**

![Figure 1: Effect of Nitrogen on Induction and Multiplication of Shoots on MS Medium +Kn (1.0 mg/l) + BAP (5.0 mg/l) + NH4NO3 + KNO3](image-url)

Figure 1: Effect of Nitrogen on Induction and Multiplication of Shoots on MS Medium +Kn (1.0 mg/l) + BAP (5.0 mg/l) + NH4NO3 + KNO3
Figure 2: Effect of Plant Growth Regulator on Induction of Roots on MS Media + NH₄NO₃ (14 N/l) + KNO₃ (400 N/l) without Activated Charcoal

Figure 3: Effect of Plant Growth Regulators on Induction & Multiplication of Roots on MS Media with NH₄NO₃ (14 N/l) + KNO₃ (400 N/l) + Activated Charcoal (0.5 g/l)

Figure 4: Effect of Nitrogen on Formation of Root on MS Basal Medium + IAA (1.0 mg/l) + ß-NAA (1.0 mg/l) + NH₄NO₃ + KNO₃