ESTABLISHMENT OF PROFICIENT *IN VITRO* MASS MULTIPLICATION AND REGENERATION SYSTEM FOR ENHANCED PRODUCTION OF STEVIOSIDE AND REBAUDIOSIDE A IN *STEVIA REBAUDIANA*

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ABSTRACT

Stevia rebaudiana is not only a pivotal source of non-caloric sweetener but is also a medicinally important herb. Propagation through seed has certain limitations as it responds in particular environmental conditions and cannot tolerate temperature beyond 30ºC. Keeping these limitations in mind, a proficient *in vitro* micro-propagation and regeneration system was established in this sweet herb to promote it as a non-caloric sweetener. In this experiment, various concentrations and combinations of growth regulators were tested to seek for the most responsive protocol for micro-propagation and regeneration. The best regeneration response was observed on MS medium containing Murashige and Skoog salts (4.33g/L), sucrose (30 g/L), 6-benzalamino purine (1.5 mg/L) and kinetin (0.5 mg/L) with maximum number of shoots (19.00± 0.57). Similarly, indole-3-butyric acid proved to be an appropriate growth regulator for root induction and proliferation. Rather differential role of IBA was observed as root number increased but root length decreased with increase in IBA concentration. The acclimatized plants in pots were tested for the production of non-caloric sweeteners (stevioside and rebaudioside A) through High Performance Liquid Chromatography (HPLC). Hence, we conclude that this protocol will prove a step forward in promoting availability of this non-caloric sweetener for diabetic patients.

Key words: Micro-propagation, growth regulators, HPLC, qualitative analysis, stevioside, rebaudioside A.

INTRODUCTION

Changing life style and dietary habits have resulted drastic increase in diabetes and obesity. As a result, people are more calorie conscious which has uplifted demand for non-caloric sweeteners and eatables. Non-caloric sweeteners including synthetic sweeteners contribute more than 20% of total sugar consumed (Anonymous 2018). *Stevia rebaudiana* Bertoni L. is a valuable sweet herb, well known as a non-caloric sweetener. More than 150 species of stevia have been explored so far in different regions of the world (Debnath, 2008). Stevioside and rebaudioside A are the core chemical compounds responsible for sweetness of this sweet herb. These steviol glycosides have no calories or glycemic index as of table sugar so, are safe for diabetes. Further, they are 100-300 times sweeter than sucrose hence are required in much lesser quantity (Lemus-mondaca, 2012; Ramesh, 2006). In addition to diabetes and antitumor effects, this plant has valuable impacts including cure for obesity, high blood pressure, tobacco and alcohol addiction, hypoglycemia, heart burn, fungal infections and cardiac patients (Yasukawa *et al*., 2002). Presence of flavonoids and phenolic compounds makes this plant a valuable antioxidant and antimicrobial agent (Uddin *et al*., 2006). Japan is the global leader in consuming stevioside as natural sweetener where it is extensively used in food and pharmaceutical industry. Japan alone is known to import stevia or stevia products of worth $4.95 million per year. Its cultivation has now expanded to several other countries including India, China, Malaysia, Singapore, South Korea, Taiwan, and Thailand (Brandle *et al*., 2000).

Propagation of Stevia from seed has certain limitations i.e. small endosperm or infertile seed. Some of the genotypes are not able to produce viable seed because of self-incompatibility (Yadav *et al*., 2011; Raina *et al*., 2013). Even plants raised from seeds do not result in homogenous population and are highly variable in traits including sweetness (Rathi and Arya, 2009). Conventional vegetative propagation of stevia has been found inappropriate owing to pathogen infestation and poor growth response of plants (Mishra *et al.,* 2010). Under these circumstances *in vitro* micro-propagation of stevia is the only effective and reliable approach for mass multiplication and to get homogeneous plants of this sweet herb. Various concentrations and combinations of growth regulators and even explants have been explored by a number of research groups. A range of experiments have been conducted to optimize growth conditions and appropriate growth regulators. Stem nodes and shoot tips were found best responsive to micropropagation when cultured on MS medium augmented with 2.0 mg/L 6-benzyl amino purine (BAP) (Hassanen and Khalil, 2013; Aman *et al*., 2013; Khan *et al*., 2014). Nodal segments were found best responsive explants for regeneration when MS medium was supplemented with BAP and

Kinetin (Razak *et al.,* 2014) or 6-benzyl amino purine and 1-Naphthaleneacetic acid (Soliman *et al*., 2014). To conserve such an economically valuable herb, the most desirable technique is tissue culture (Cenkci *et al*., 2009). Considering the importance of this valuable sweet herb, a proficient *in vitro* mass multiplication and regeneration system was established in *Stevia rebaudiana*.

MATERIALS AND METHODS

Surface sterilization of ex-plant: Seeds were collected from Ayub Agricultural Research Institute, Faisalabad. They were surface sterilized by successive immersions in tap water, 70% (v/v) ethanol for 2 minutes followed by 60% commercial bleach with few drops of tween 20 for half an hour while on shaking. Then seeds were rinsed 3-4 times with sterile distilled water and were cultured in Petri plates and glass test tubes containing MS0 medium (Shojaei *et al.*, 2010). The cultured samples were placed in growth room at 25°C ± 2°C undercool white fluorescent illumination 16:8-hour light and dark condition.

Shoot induction and proliferation: Nodal segments taken from *in vitro* growing plants were cut into small pieces of ≈0.51 cm and were cultured on MS medium augmented with various concentrations and combinations of growth regulators. Different types of regeneration media (RM1, RM2, RM3, RM4, RM5, RM6) were tested to seek the optimistic one conducive for prompt regeneration (Table 1). The basal medium without hormones was taken as control. Five to six explants were placed in each petri plates (a total of three petri plates were cultured for each treatment) followed with incubation in a growth room with 2000-2500 lux day intensity with 16:8 hrs light dark regime for a period of 4 weeks. After induction of primary shoots, explants were sub cultured on the same medium and were incubated under same growth conditions as mentioned earlier.

Table 1 Different media combinations for shoot regeneration

Sr. No.	Treatment	Media composition
1	RM1	MS + BAP+ Kinetin (0.5 mg/L+ 0.5 mg/L)
2	RM2	MS + BAP + Kinetin (1 mg/L + 0.5 mg/L)
3	RM3	MS+ BAP + Kinetin (1.5 mg/L + 0.5 mg/L)
4	RM4	MS + 0.5 mg/L BAP
5	RM5	MS + 1 mg/L BAP
6	RM6	MS + 1.5 mg/L BAP

Root induction in *in vitro* established plants for enhanced acclimatization: Four weeks old *in vitro* grown shoots were cultured on different concentrations of Indole-3-butyric acid (IBA). Six different levels of IBA (0, 1, 2, 3, 4, 5 and 10 mg/L) were used. Root length as well as total number of roots were determined after four weeks of culture on rooting medium.

Preparation of sample for HPLC: Fresh mature green leaves were harvested from acclimatized *Stevia rebaudiana* plants. They were washed with distilled water and dried at room temperature. The leaves were blended in blender to make powder and were stored at 4 °C. The ground fine powder (5 gm) was used for extraction. For this, leaf powder was mixed with 50 ml methanol in Soxhlet apparatus for 2 hours. The extract was filtered (using Whatmann No. 1 filter paper) and the residues were re-extracted twice with methanol. It was evaporated at 60°C and was re-dissolved in 50 ml double distilled autoclaved water to prepare final extract. Then extract was treated with diethyl ether (25 ml) to remove photosynthetic pigments. Lower transparent layer was collected and mixed with butanol. Then upper layer was collected and was used for HPLC (reverse phase chromatography) analyses. Shim-Pack CLC-ODS (C-18) column with dimensions LxW (25 cm × 4.6 mm) and pore size 5 µm was used. Column temperature was maintained at 28ºC and samples were analyzed at wavelength of 245 nm. HPLC grade acetonitrile with water (80:20) at pH 3.0 along with 85% reagent grade phosphoric acid was used as mobile phase. The samples were filtered with 0.22 µm millipore filter whereas injection volume was set to 10 µL at a flow rate of 1 ml/min.

Data collection and analyses: Data were collected considering various parameters i.e. frequency of shoots from *in vitro* growing plantlets, root induction frequency including number of roots and root length. A completely randomized design (CRD) with three replicates per treatment was used in these studies (Steel *et al*., 1997). Data were analyzed by analysis of variance for significance using statistix 8.1 software. Means were compared using least significant difference (LSD) test for significance.

RESULTS

Establishment of proficient *in vitro* regeneration: Eight to ten weeks old *in vitro* grown plants were used as explant for the establishment of proficient *in vitro* regeneration. After 5-6 days of culture, shoots appeared from the nodes. Among different types of culture media (RM1, RM2, RM3, RM4, RM5 and RM6), RM3 appeared to be most influential. More than 19 ± 0.57 shoots regenerated after four weeks of culture. Other media combinations also responded as regeneration was almost same on RM1, RM2 and RM4 which resulted in 5.33 ± 0.33, 6.66 ± 0.33 and 6.0 ± 0.57 shoots respectively. Shoot length also appeared to be maximum at RM3 (7.1 ± 0.32) followed by RM5 (4.23 ± 0.08), RM6 (4.23 ± 0.14), RM2 (4.16 ± 0.17), RM1 (3.46 ± 0.14) and RM4 (3.26 ± 0.12) cm. Hence, RM3 appeared to be the best combination of growth regulators as far as shoot induction and proliferation is concerned (Figure 1) and may be used for the proficient mass multiplication of this valuable source of non-caloric sweetener.
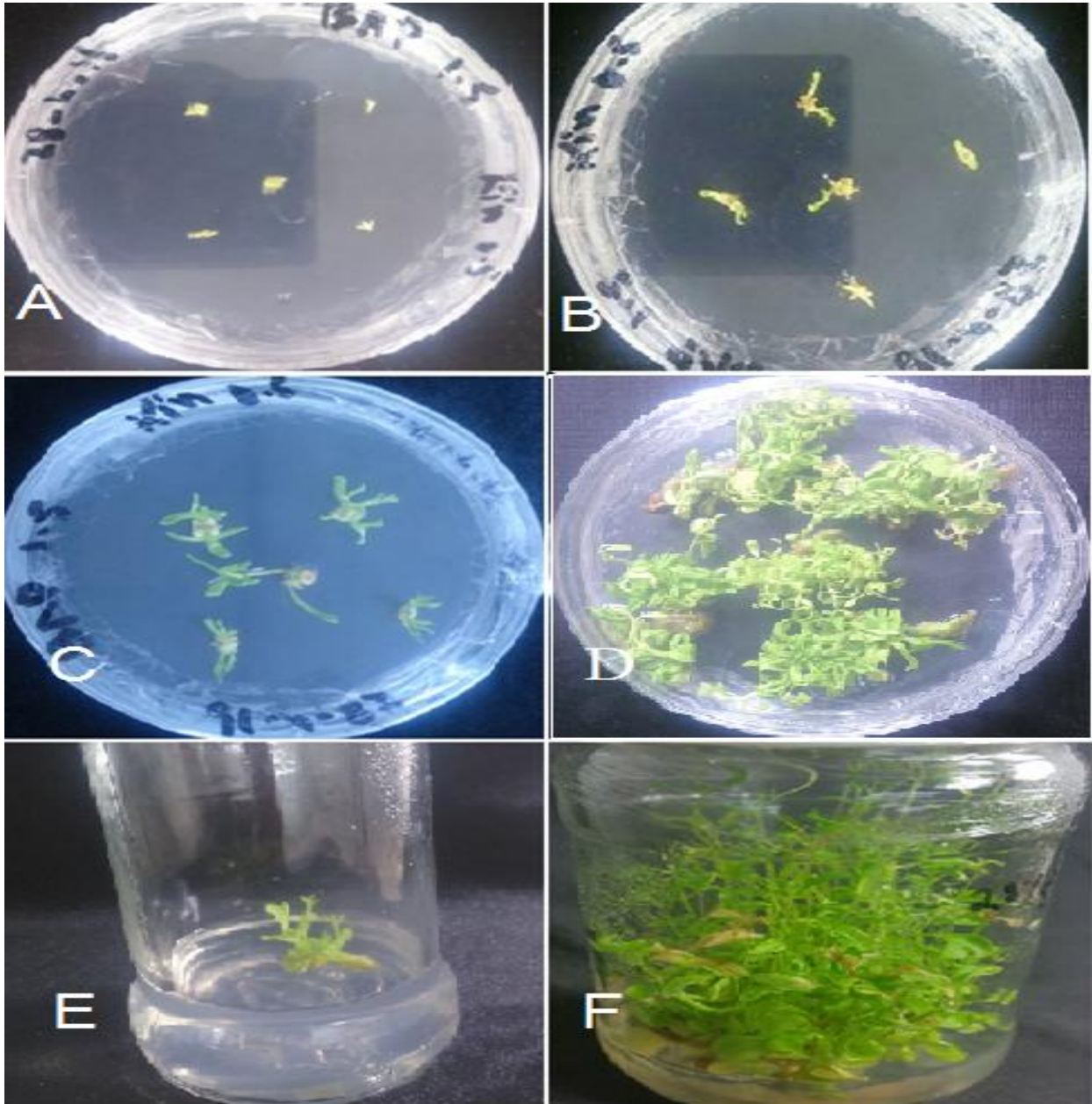

Figure 1. Shoot proliferations from nodal segments on RM3 medium. A) after 1ˢᵗ day of culture B) after 5 days of culture C) after 10 days of culture D) after 15 days of culture E) regenerated shoots shifted to jar F) after one month of shifting to jar.

Table 2. Mean values for shoot length and number of shoots regenerated from nodes.

Medium combination	Mean shoot length (cm) ± SE	Mean number of shoots ± SE
RM1	3.46± 0.14C	5.33 ± 0.33C
RM2	4.16± 0.17B	6.66± 0.33C
RM3	7.10± 0.32A	19.00± 0.57A
RM4	3.26± 0.12C	6.00± 0.57C
RM5	4.23± 0.08B	9.00± 0.57B
RM6	4.23± 0.14B	9.66± 0.33B

Differential role of IBA in root induction and proliferation: For proficient rooting in *in vitro* established plants, six different levels of IBA (0.0, 2.0, 3.0, 4.0, 5.0 and 10.0 mg/L) were used. Data analyses revealed that root length decreased with increase in IBA concentration whereas root number decreased with increase in IBA concentration. Maximum root length (1.26±0.081 cm) was observed in control plants growing on IBA free medium followed by plants growing on 1.0, 2.0, 3.0 and 4.0 mg/L of IBA, where root length was determined as 0.92±0.037, 0.50±0.04, 0.40±0.07, and 0.50±0.03 cm respectively. Plants growing on 5.0 and 10.0 mg/L of IBA were not able to survive since higher dose of IBA (5.0 and 10.0 mg/L) appeared to be detrimental for plant growth. Contrarily, root number increased with increase in IBA concentration and was determined as 7.30± 0.31, 9.60± 0.50, 14.20± 0.37, 9.5.00± 0.70 and 12.00± 0.37 at 0, 1, 2, 3 and 4 mg/L respectively (Figure 2). Hence, IBA treatment appeared to promote root induction and proliferation.
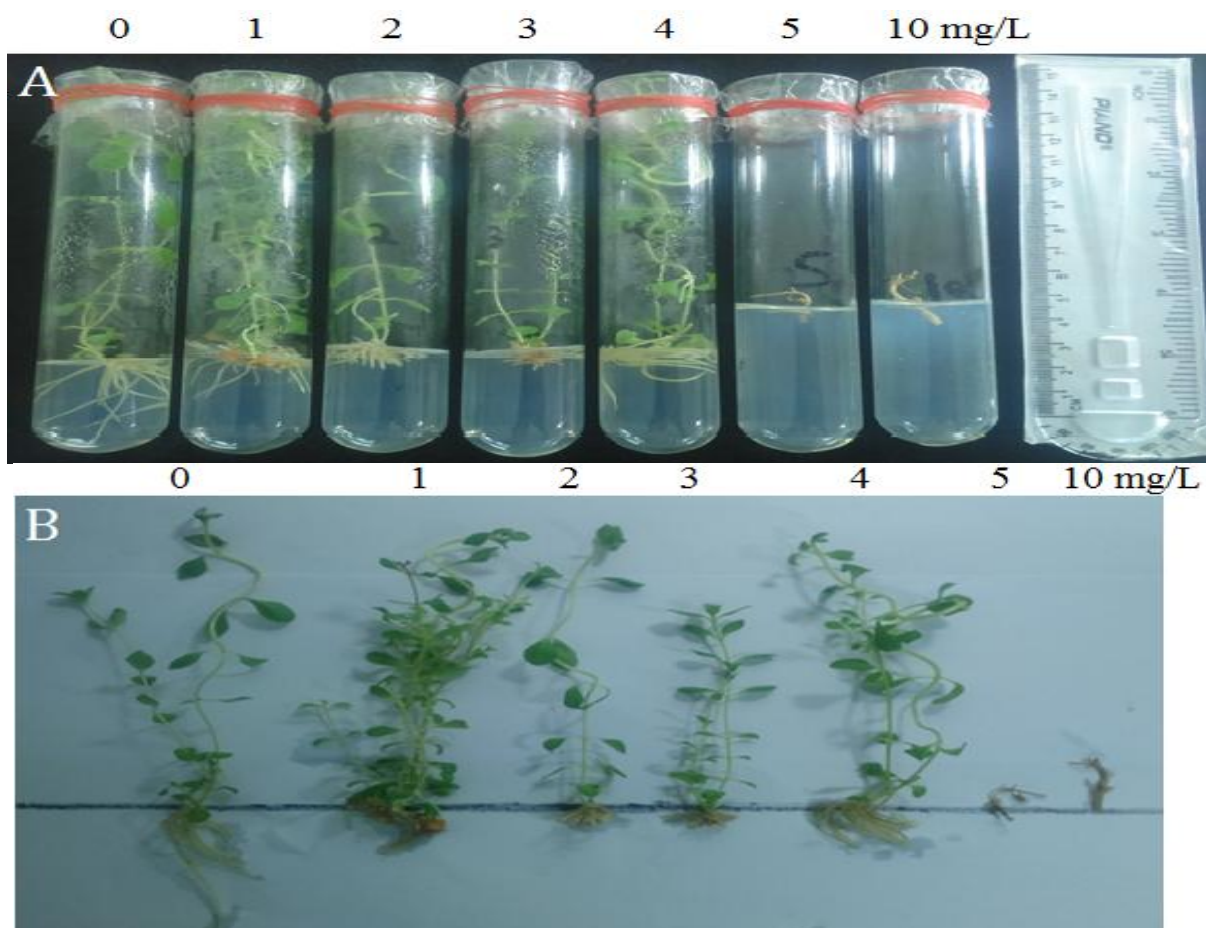

Figure 2. Effect of Indole-3-butyric acid on root development in *Stevia rebaudiana*. A). Root induction and growth on MS medium supplemented with different concentrations of IBA ranging from 0.0, 1.0, 2.0, 3.0, 4.0, 5.0 to 10.0 mg/L. B).

Qualitative analysis of stevioside and rebaudioside A by High Performance Liquid Chromatography: High performance liquid chromatography (HPLC) is a very sensitive and precise technique for the estimation of rebaudioside A and stevioside contents in the desired samples. Prepared leaf samples of *Stevia rebaudiana* were analyzed and retrieved chromatogram showed detection of stevioside and rebaudioside A. Retention time (Rt) for stevioside was 1.687 min whereas for rebaudioside A, it was 2.020 min with solvent flow rate

of 1 ml/min (Figure 3), detection wavelength 245 nm and column of Shim-Pack CLC-ODS (C-18), 25 cm × 4.6 mm, 5µm. The determined retention time validated prevalence of stevioside and rebaudioside A in the leaves of *in vitro* propagated plants.
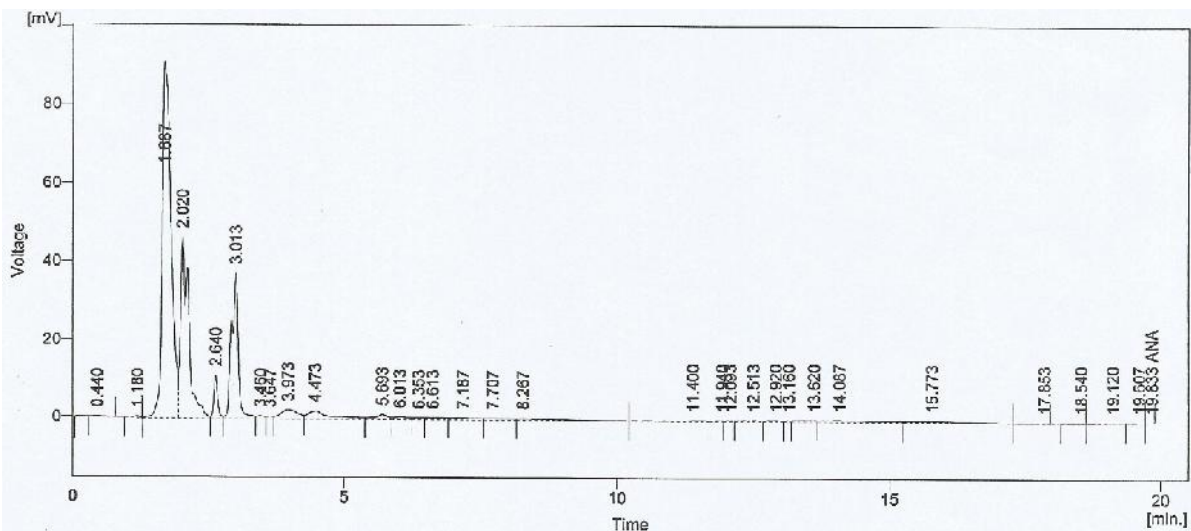

Figure 3. HPLC Chromatogram for stevioside and rebaudioside A. Retention time (Rt) for stevioside was observed to be 1.687 min whereas for rebaudioside A as 2.020 min.
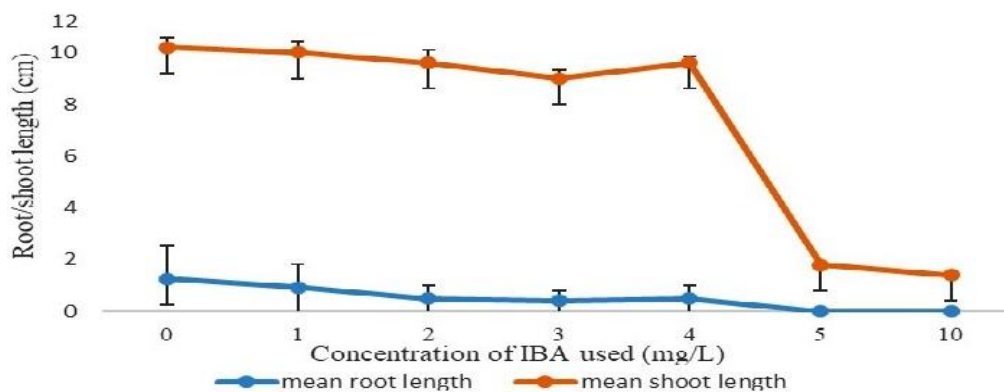

Figure 4. Mean shoot and root length at different concentrations of indole-3-butyric acid. Maximum shoot length was observed at 1 mg/L IBA whereas maximum root length was observed on MS medium without IBA.
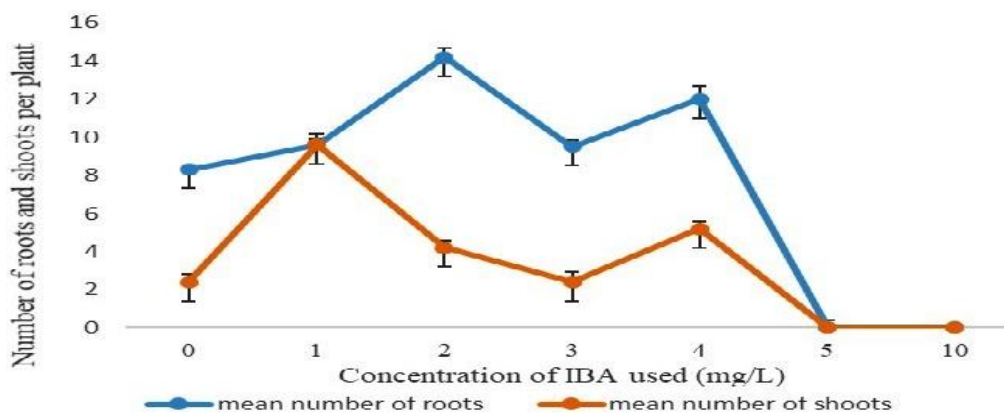

Figure 5. Mean number of roots and shoots at different concentrations of indole-3-butyric acid. Maximum number of shoots was observed on 1 mg/L IBA. Likewise, maximum number of roots were observed on MS medium containing 2 mg/L IBA.

DISCUSSION

Stevia rebaudiana, a sweet herb, is grown all over the World for its sweet leaves. The steviol glycosides are used in tea, beverages, cold drinks, juices, pastries, bakery products and processed diets. These glycosides have zero calories and can be used as sucrose substitute in low calorie foods. Stevia has beneficial effects for diabetics, patients of blood pressure and obese persons (Das *et al*. 2011). Different protocols have been given by various research groups for the establishment of mass multiplication and regeneration system in this sweet herb. Since BAP has appeared to be the most valuable growth regulator for shoot induction or regeneration. Ahmad *et al*. (2011) reported maximum shoot induction per explant when used at a concentration of 1.0 mg/L. Francis *et al*. (2007) also evaluated 1.0 mg/L as optimal concentration of the BAP for regeneration. Aman *et al*. (2013) obtained highest number of shoots on MS medium augmented with 1.0 mg/L BAP whereas maximum number of leaves were obtained on MS medium augmented with BAP (1.0 mg/L) and kinetin (1.0 mg/L). We observed maximum number of shoots (19.00±0.57) at 1.5 mg/L BAP with 0.5 mg/L kinetin (Figure 5). Likewise, maximum shoot length was also observed on the same RM3 medium. Hence, we evaluated that BAP in combination with kinetin gives better results as far as mass multiplication of stevia is concerned. In addition to stevia, BAP has also proved to be an effective growth regulator for regeneration and mass multiplication in other crop species (Huii *et al*., 2012; Badou *et al*., 2018).

Indole-3-butyric acid plays key role in root induction and proliferation. Mustafa and Khan (2016) used six concentrations of IBA and found maximum rooting at 5 mg/L IBA. Further, they evaluated differential role of the auxin in root development. Likewise, we also observed that root number increased with increase in IBA concentration (1 mg/L to 4 mg/L). Afterwards, it appeared to be decreased with increase in IBA concentration (5 mg/L and 10 mg/L). Thus IBA @ 4 mg/L appeared to be the most appropriate dose for proficient rooting. At the same time, higher (5 and 10 mg/L) concentration of IBA appeared to have negative effect on shoot elongation as was reported by Dey *et al*. (2013).

HPLC is normally used for the qualitative analysis of the sweeteners stevioside and rebaudioside A. Afandi *et al*. (2013) reported retention time (Rt) for stevioside and rebaudioside A as 3.3 min and 4.1 min respectively whereas Shirwaikar *et al*. (2011) determined retention time for stevioside as 10.500, 10.683, 10.717 in case of commercial stevia powder and 10.075 min for leaf stevia extract. We observed Rt values for stevioside and rebaudioside A as 1.687 and 2.020 min respectively which validated presence of these non-caloric sweeteners in this sweet herb. Hence, reported protocol may be used for time proficient and economical mass multiplication of *Stevia rebaudiana* L. as a result, cheaper sweet herb powder will be available to diabetic patients.

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