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EFFECT OF NITROGEN ON MICROPROPAGATION OF *Stevia rebaudiana* (BERTONI)

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ABSTRACT

In the present study, the effect of nitrogen was investigated on the micro propagation of *Stevia rebaudiana* to overcome the challenges related to its cultivation. The *in vitro* shoots were cultured on MS medium fortified with BAP (5.0 mg/l) along with 2,4-D (0.1 mg/l). Four weeks old multiple shoots were transferred on modified MS medium containing inorganic nitrogen sources i.e NH_4NO_3 (14-56 N mg/l), KNO_3 (100-400 N mg/l) with BAP (0.5-5.0 mg/l) were observed. Twelfth weeks cultures recorded maximum multiplication of shoots on MS medium with NH_4NO_3 (14 N mg/l), KNO_3 (400 N mg/l) with BAP (5.0 mg/l). Moreover 90% of them were able to re-grow when sub-cultured on same media. Twelve weeks old cultures multiple shoots were transferred on MS medium with IAA (1.0 mg/l) for rooting find out to be best. Five percent cultures showed hairy root growth. Survival of 85% was achieved when rooted plantlets were acclimatized in 2 FYM: 1 Perlite: 1 Garden Soil.

INTRODUCTION

The composition of the culture media is one of the major components which accounts for the specific results of plant tissue culture. Nitrogen is being the major component in almost all media plays an important role in plant growth and development. Both the form and amount of nitrogen in the *in vitro* medium have significant effects on rate of cell growth, differentiation and cell totipotency (Kirby *et al*., 1987). The potential benefits of optimizing the nutrient components of culture media for a particular response are well reported across a wide range of plant species. The concentration of NH_4^+ and NO_3^- affects numerous *in vitro* responses including the development of somatic embryos (Elkonin and Pakhomova 2000; Leljak-Levanić *et al*., 2004; Zafar 2005) the efficiency of plant recovery after ovule culture (McCoy and Smith 1986), shoot regeneration, (Leblay *et al*., 1991; Vinterhalter *et al*., 2007) regulation of growth and biomass of bioreactor-grown plantlets, (Sivakumar *et al*., 2005) and also controls the rate of root initiation in shoot cultures. In the last couple of decades, growing concern about health and quality of life has encouraged consumer's interest in reducing sugar intake, food products made with sweeteners rather than the sugar. Sweeteners are alternative substances to sugars, which give food a sweet taste and are used to partially or totally replace sucrose. One of such natural, non-nutritive sweeteners with zero calorie is *Stevia rebaudiana* (Bertoni) that belongs to family Asteraceae and its sweet leaves are used to impart sweetness to foods through steviol glycosides i.e. Stevioside and Rebaudioside A, secondary metabolites of the *Stevia rebaudiana* (Kinghorn *et al*., 1984). Besides its sweetening properties it also accounts for many medicinal properties such as anti-hyperglycaemic, anticancerous (Jeppesen *et al*., 2002, 2003) and anti-hypersensitive (Jeppesen *et al*., 2003) without any side effect. Reported reasonably sound track record of plant constituents, particularly *Stevia rebaudiana* (Steviosides, glycoside) as intense sweetening agent and because of the great public demand for natural food ingredients, especially for diabetic and dietetic applications as large biomass production is required but propagation of the plant through natural methods is not possible as seed germination of this plant is very low and great variation in features of plant secondary metabolites thus produced is recorded (Nakamura and Tamura 1985). Keeping all above mentioned aspects in consideration the present study was designed to investigate effect of nitrogen on micro propagation of *Stevia rebaudiana* (Bertoni).

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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 (Murashige and Skoog 1962) 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_4NO_3 (14-56 N/l) + KNO_3 (100-400 N/l) 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.

RESULT AND DISCUSSION

Average number of buds and length of shoots (in cm) were recorded for *in vitro* shoots as an explant in micro propagation of *Stevia rebaudiana.* The use of nodes from multiplied shoots as an explant was in line with some previous studies (Banerjee and Sarkar 2008) where leaf , nodal segment and internodes were taken as explant and nodal was proved to produce faster callogenesis as compared to other explants while leaves as an explants proved to be best explants for callus induction response in studies of (Arya *et al*., 2012). Micro propagation cultures showed that plant growth regulators has an essential impact on the shoot induction as well as on its multiplication. The present study was undertaken with the two cytokinins, BAP and kinetin (Kn). BAP proves to be having much more prudent impact on shoot induction as well as on proliferation as compared to Kinetin. These results were comparable with previous finding where BAP was reported as best hormone for shoot formation in Stevia (Ali *et al*., 2010). BAP 5.0 mg/l alone showed maximum on shoot induction with maximum average number of buds 4.88 ± 1.08 while maximum shoot length (5.78 ± 1.20 cm) was achieved with 2.0 mg/l BAP alone (

Graph 1). Kinetin (0.5- 3.0 mg/l) was used for the same parameters. Kinetin alone at concentration 3.0 mg/l showed maximum 3.31 ± 0.98, 3.44 ± 0.98cm average numbers of buds and average shoot length respectively and these results were in line with (Tadhani and Subhash 2006). Further the combination of both the cytokinins BAP (3.0- 5.0 mg/l) and Kn (1.0- 3.0 mg/l) was taken to study the effect of two parameters in study. The combination of BAP (5.0 mg/l) + Kn (1.0 mg/l) was proved to be best combination for the average number of buds 5.17 ± 1.17 and length of shoot was 4.34 ± 1.23 cm (

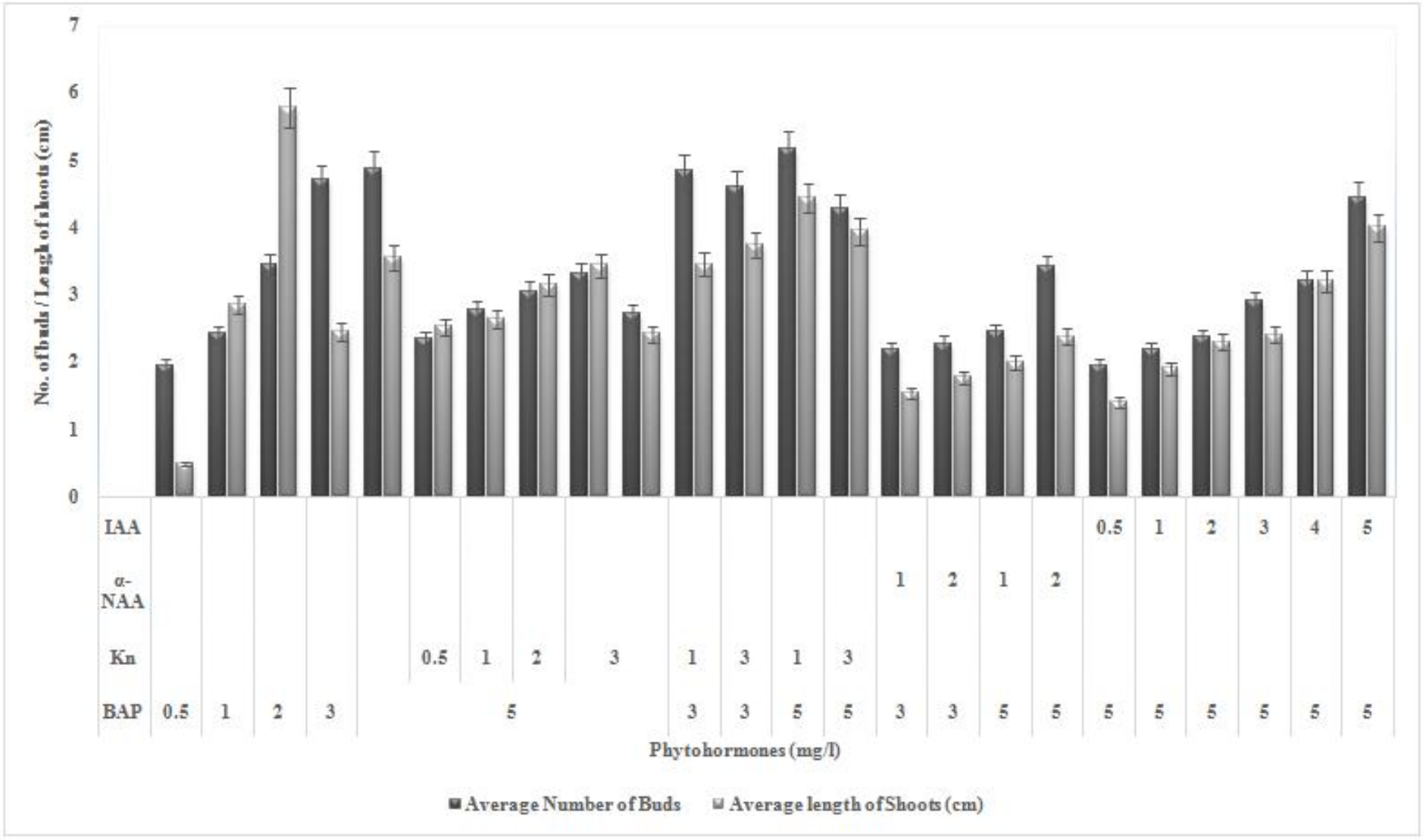
Graph 1). Our findings also advocated that better results for shoots for their induction as well as multiplication can be achieved when media is supplemented with both BAP and Kn.

These results were supported by Bhatt and Dhar (2000) and Ahmed *et al*. (2007) for the shoot induction. Present result is also in consistence with Cononer and Litz (1978) in Papaya and Teixeira and Da Silva (1990) in eucalyptus. BAP (1.0 and 3.0 mg/l) was used for the further response in combination with auxins, α-NAA (1.0 and 2.0 mg/l) and IAA (0.5 – 5.0 mg/l). BAP (5.0 mg/l) + IAA (5.0 mg/l) showed maximum response of average number of buds 4.45 ± 3.88 and average length of shoot 4.0 ± 1.01 cm for shoot induction while the observations recorded for above mentioned two parameters during BAP (5.0 mg/l) + α- NAA (2.0 mg/l) media supplementation were 3.41 ± 0.73, 2.38 ± 0.07cm respectively. The combination of BAP with IAA showed better shoot induction as compared with the combination of BAP with α-NAA. These results showed similarity with the reports of Debnath (2008). Their study reported that MS medium supplemented with BAP (2.0 mg/l and IAA (1.13 mg/l) in combination were found to be most effective in initiation of multiple shoot proliferation. The effect of ammonium nitrate and potassium nitrate was tested on the process of multiplication in micro propagation of *Stevia rebaudiana* (

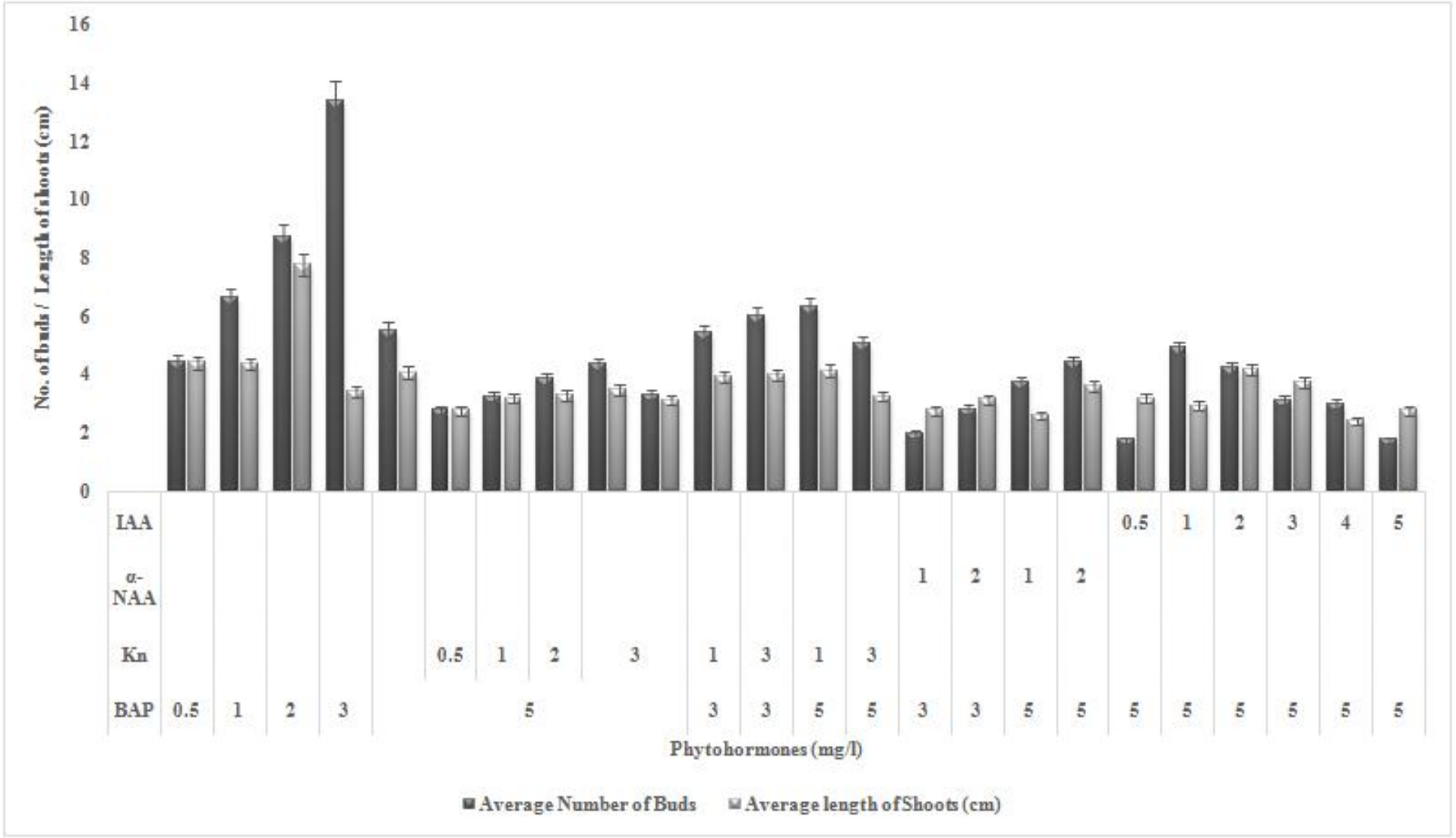
Graph 2). The use of inorganic nitrogen for plant development was also in alignment with Ramage and Williams (2002) results from that study showed a synergistic effect between ammonium and nitrate on shoot organogenesis independent of pH. Ammonium nitrate (14 N mg/1 to 56 N mg/1) and potassium nitrate (100 N mg/1 to 400 N mg/1) were added in combination and results were recorded at three weeks intervals. Up to twelfth week of culture, the length and the number of shoots were found to be increased.

In the third week maximum number of shoots and shoot length was produced on MS medium + BAP (5.0 mg/l) supplemented with the combination of NH_4NO_3 + KNO_3 (14 N mg/1 + 400 N mg/1) that was 24.30 ± 0.81, 2.52 ± 0.17 respectively. The texture of shoots varied between green to light green. In the sixth week observations again the maximum number of shoots and shoot length was produced on MS medium+ BAP (5.0 mg/l) supplemented with the combination of NH_4NO_3 + KNO_3 (14 N mg/1 + 400 N mg/1) 53.2 ± 1.50, 4.22 ± 0.26 cm respectively (

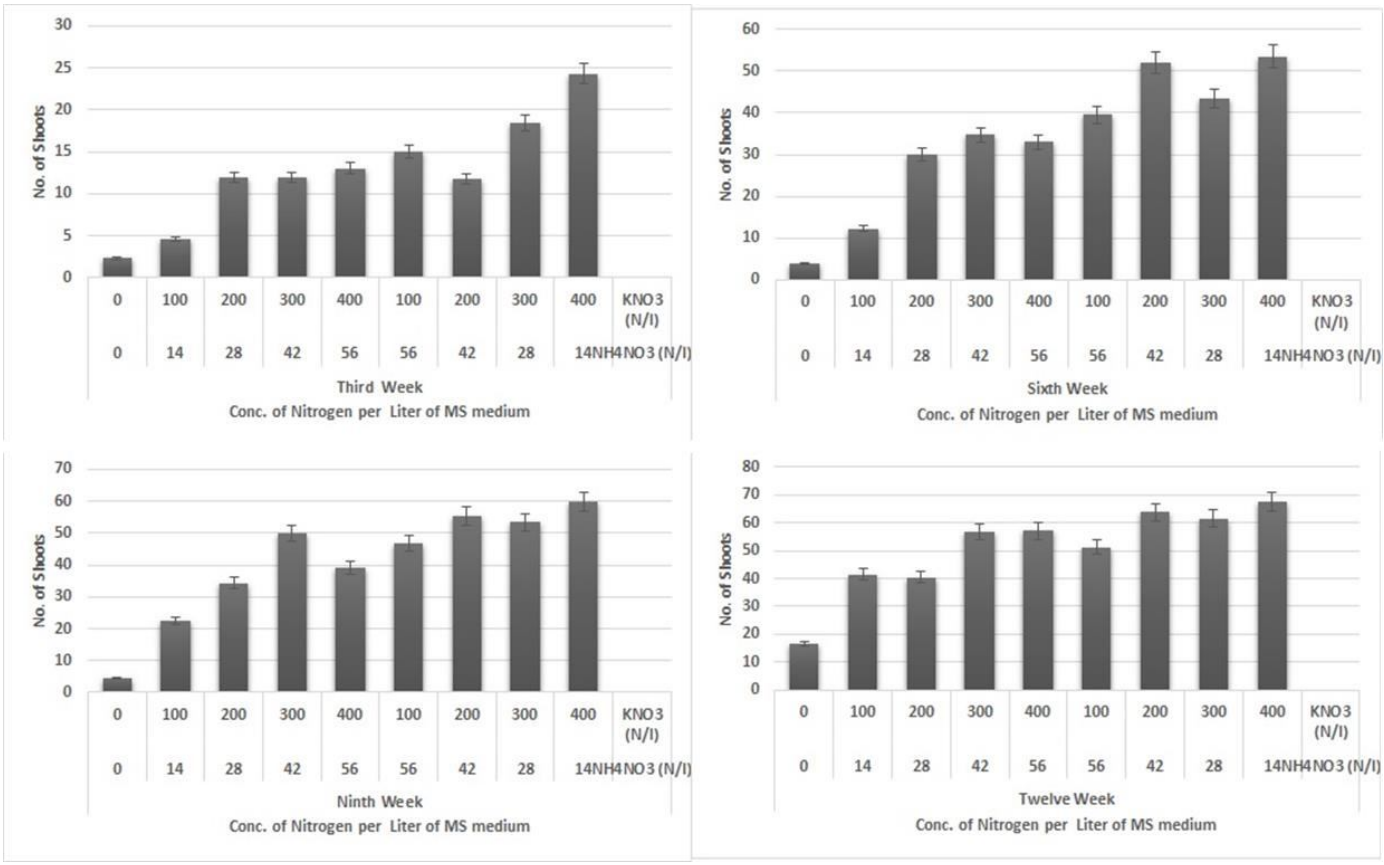
Graph 3, Graph 4). The texture of shoots varied between green to light green. In the ninth week observations again the maximum number of shoots and shoot length was produced on MS medium supplemented with the combination of NH_4NO_3 + KNO_3 (14 N mg/1 + 400 N mg/1) 59.5 ± 1.30, 6.10 ± 0.19 cm respectively. The texture of shoots varied between green to whitish green. However these results were comparable with (Rahman *et al*. 2011). In the twelfth week observations again the maximum number of shoots and shoot length was produced on MS medium supplemented with the combination of NH_4NO_3 + KNO_3 (14 N mg/1 + 400 N mg/1) 67.0 ± 0.45, 7.3 ± 0.23 cm respectively (Graph 4).



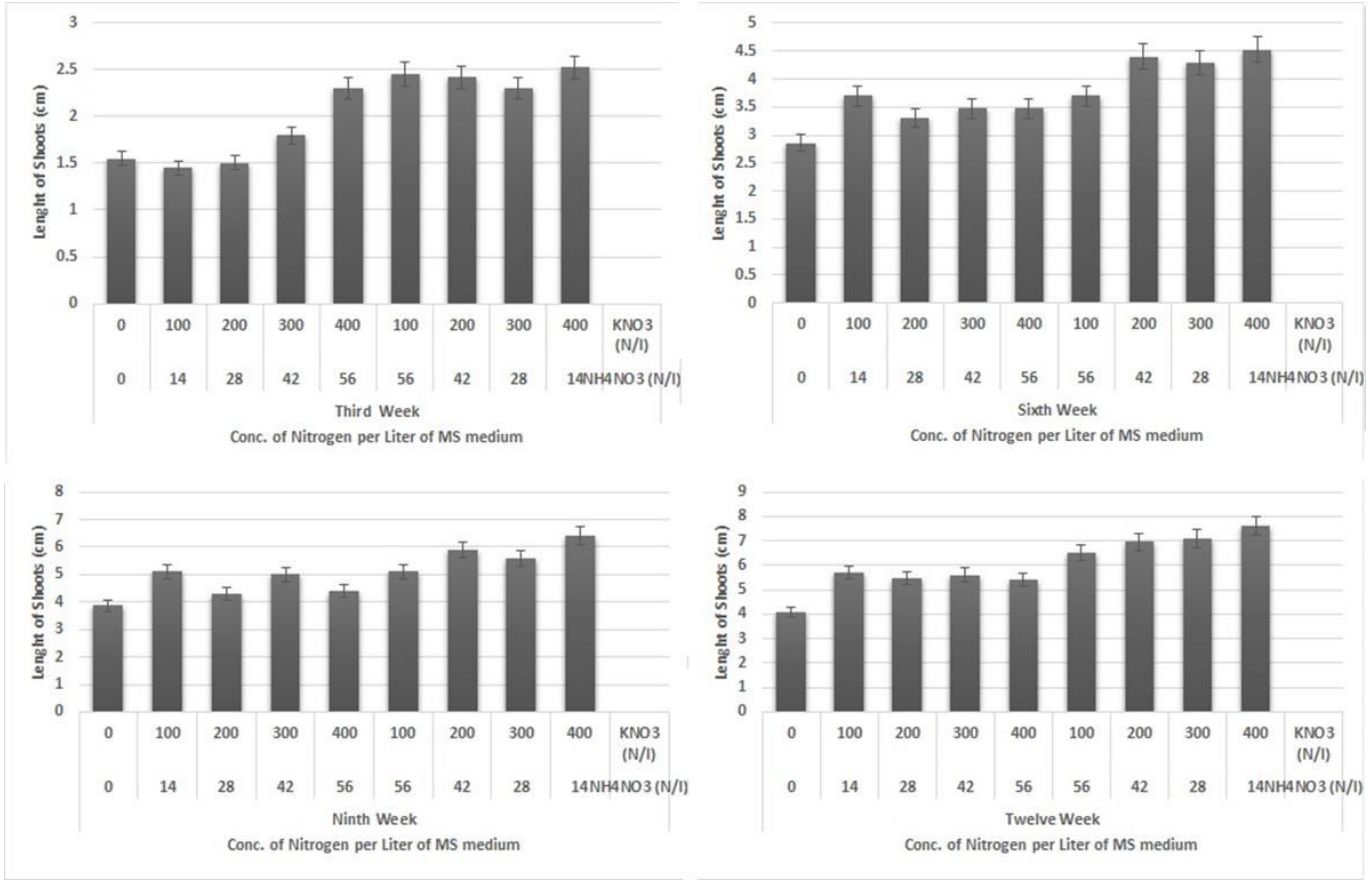
Graph 1: Effect of cytokinins and auxins combination supplemented in MS medium on induction of axillary buds on *in vitro* shoots of *Stevia rebaudiana*. Data recorded after 4 weeks of culture



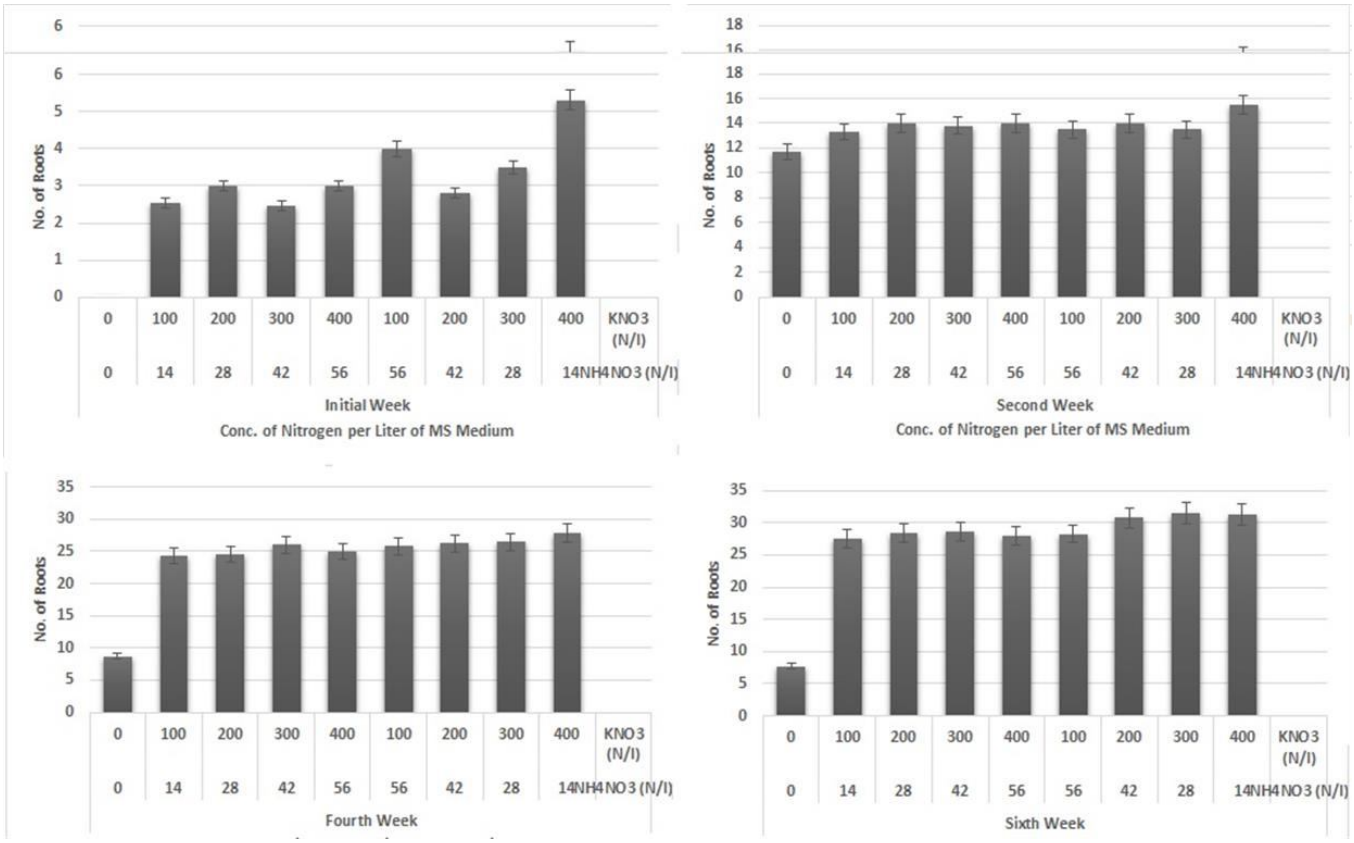
Graph 2: Effect of cytokinins and auxins combination supplemented in MS medium on multiplication of *in vitro* raised shoots of *Stevia rebaudiana*. Data recorded after 4 weeks of culture



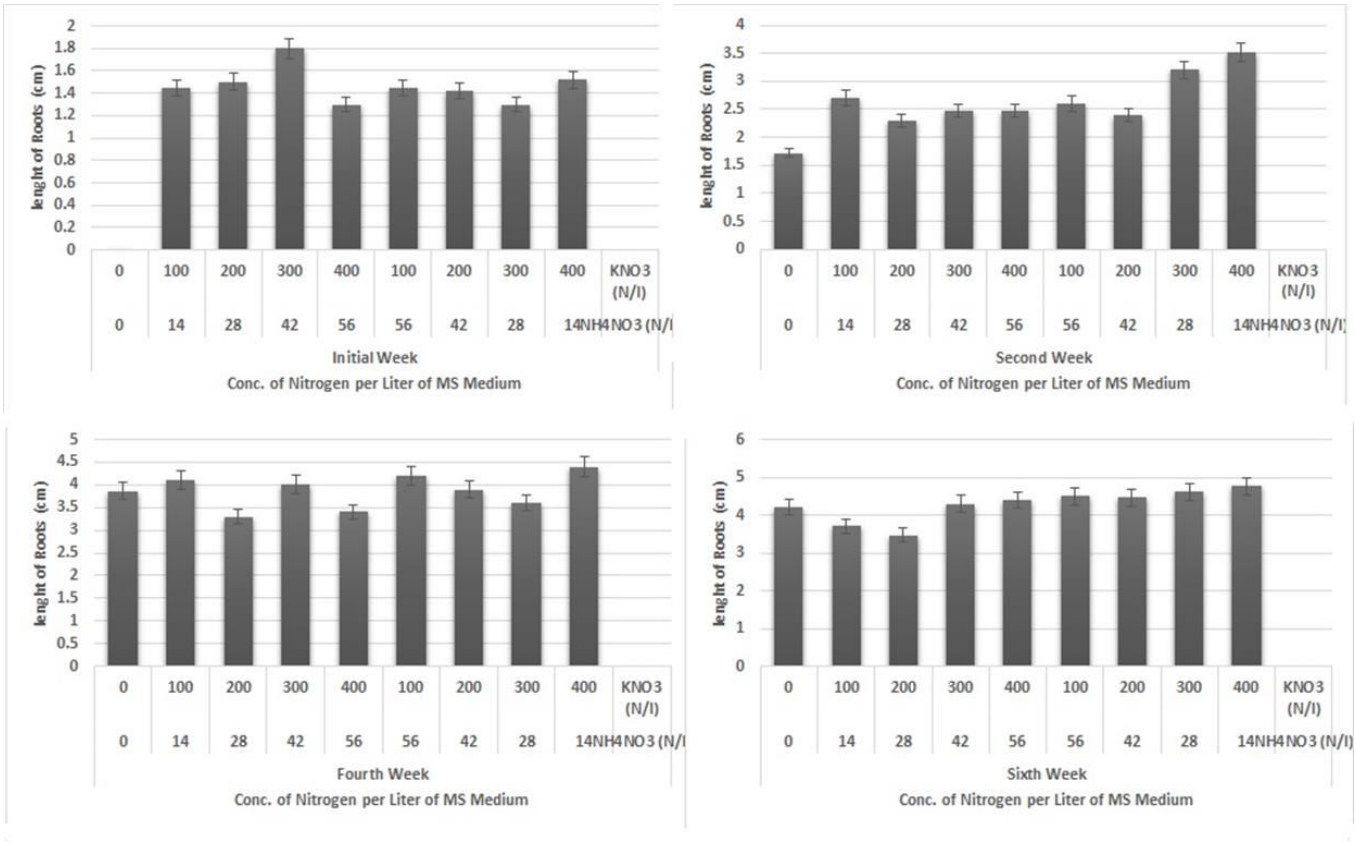
Graph 3: Effect of Nitrogen on Number of shoots during multiplication on MS with BAP (5.0 mg/l) of *Stevia rebaudiana*



Graph 4: Effect of Nitrogen on Length of shoots during multiplication on MS with BAP (5.0 mg/l) of *Stevia rebaudiana*



Graph 5: Effect of Nitrogen on Number of Roots during multiplication on MS Basal medium + IAA (1.0 mg/l) + α-NAA (1.0 mg/l) + NH_4NO_3 + KNO_3



Graph 6: Effect of Nitrogen on Length of Roots during multiplication on MS Basal medium + IAA (1.0 mg/l) + α-NAA (1.0 mg/l) + NH_4NO_3 + KNO_3

These results were also supported by Villamor (2010) the texture of shoots was green. The minimum number and length of shoot was observed when media was not supplemented with any nitrogen source. These results showed a co-ordination with Sen and Batra (2011) they reported that there was no morphological changes and growth when *Phyllanthus amarus* explants were cultured on MS medium devoid of nitrogen sources. It was found that media with higher nitrogen content was much better for plant *in vitro* regeneration. The various combination of nitrogen on the formation of roots on MS medium along with IAA (1.0 mg/l) and α- NAA was recorded (

Graph 5, Graph 6). In initial week maximum number of roots (5.0 ± 0.81) was observed with 42 N/l, 300N/l and maximum length (1.5 ± 0.47 cm) of root was observed with 14 N/l, 400N/l of NH_4NO_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 14N/l, 400N/l of NH_4NO_3 and KNO_3 (

Graph 5). In fourth week increased number of roots (27.5 ± 1.30) and increased length (4.1 ± 0.19 cm) of root was observed with 14N/l, 400N/l of NH_4NO_3 and KNO_3. In sixth week maximum number of roots (31.2 ± 0.88) was recorded with concentrations 28N/l, 300N/l and increased length (4.46 ± 0.23cm) of root was recorded with 14N/l, 400N/l of NH_4NO_3 and KNO_3 (Graph 6). The use of nitrogen combinations to develop healthy root system was also advocated that root formation was much better in media devoid of NH_4NO_3 (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.

Conclusion

The results of present study clearly indicates the diverse responses to the various forms of inorganic nitrogen supplied, representing the earlier reported importance of this nutrient in determining plant morphology and morphogenesis *in vitro*.

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