

EVALUATION THE EFFECTS OF MEDIA AND PLANT GROWTH REGULATORS ON STEVIA REBAUDIANA MICROPROPAGATION

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Abstract

In order to Stevia rebaudiana micropropagation, the single node explants were cultured on MS, B5 and N6 media supplemented with TDZ (0, 0.1, 0.2, 0.3, 0.6 and 0.9 mg/l), Kin and BAP (0, 1, 2 and 3 mg/l). In this study, MS and B5 enriched with Kin and TDZ resulted to shoot induction, but BAP treatment led to callus induction. In N6 medium, single node cuttings showed malformed growth. The results revealed that MS medium supplemented with 0.9 mg/l TDZ is the most suitable treatment for in vitro micropropagation of Stevia.

Key words: Kinetin, Micropropagation, Single node, TDZ

1. INTRODUCTION

Stevia (*Stevia rebaudiana* Bertoni) is a popular member from Asteraceae family. Stevia is known as sweetest plant in the world (300 times sweeter than sugar) and most useful alternative to artificial sweeteners and sugar (Richman et al. 1999). The sweetening compounds of leaves composite of rebaudioside A, B, C, D, E and F, steviolbioside, rubsocide, dulcoside and stevioside (Starratt et al. 2002).

Stevia seeds show very low germination rate and vigor. In addition, its propagation by seeds leads to diversity in sweetness level and chemical compositions, which are limiting factors to cultivate stevia on a large scale. On the other hand, asexual propagation techniques such as stem cutting produce heterogeneous plant population and limited number of cuttings can be obtained from a single plant. Therefore, tissue culture is used in order to produce a homogeneous population of stevia at a short period of time (Miyagawa et al. 1986; Banerjee & Sarkar 2008). There are several reports on propagation and proliferation of stevia by *in vitro* culture methods with using different concentrations of plant growth regulators and explants (Yang et al. 1981; Ahmed et al. 2007; Fatima & Khan 2010; Anbazhagan et al. 2010; Gupta et al. 2010; Das et al. 2011; Jitendra et al. 2012).

Cytokinins play different roles in plants. They usually inhibit or delay root formation (Ben-Jaacov et al. 1991), prevent root growth, migrate into lateral buds and stimulate cell divisions of apical meristem. These newly activated meristem cells produce auxin which is then transported from apical buds toward downstream. This phenomenon causes to lengthen of lateral buds (Starr et al. 2012) and root development (Overvoorde et al. 2010).

Therefore, the current study was designed to investigate the effect of thidiazuron (TDZ), Kinetin (Kin) and BAP on nodal shoot explants, incubated on MS, B5 and N6 media in order to find a practical method for *in vitro* mass propagation of Stevia.

2. MATERIALS AND METHODS

2.1. Materials and procedures

The stevia seeds (purchased from JG Group Company, Canada) were sterilized with 2.5% sodium hypochlorite solution for 5 minutes and then washed three times with sterilized distilled water under laminar air flow. These seeds were transferred on Murashige and Skoog's (MS) medium (Murashige & Skoog 1962). The cultures were incubated in 25±2°C under 16 h light and 8 h dark. After germination, the plantlets were sub-cultured. After one month, single node explants containing axillary bud prepared from plantlets.

In first experiment, nodal segments were incubated on MS medium containing 0.8% (w/v) agar supplemented with various concentration of Kin (0, 1, 2 and 3 mg/l), BAP (0, 1, 2 and 3 mg/l) and TDZ (0, 0.1, 0.2 and 0.3 mg/l). This experiment was conducted based on completely randomized design (CRD) with four replications. Based on the results, we designed another CRD test with five replications. In this test, the nodal segments were incubated on MS medium containing Kin (0, 1, 2 and 3 mg/l) and TDZ (0, 0.3, 0.6 and 0.9 mg/l).

In second experiment, single node explants were cultured on B5 medium containing 0.8% (w/v) agar, Kin (0, 1, 2 and 3 mg/l), BAP (0, 1, 2 and 3 mg/l) and TDZ (0, 0.1, 0.2 and 0.3 mg/l). This experiment performed based on completely randomized design with four replications. The cultures were incubated in $25\pm 2^{\circ}\text{C}$ under 16 h light and 8 h dark. With consideration of the results, we designed another test. In this test, the nodal segments were incubated on B5 medium enriched with Kin (0, 1, 2 and 3 mg/l) and TDZ (0, 0.3, 0.6 and 0.9 mg/l). Data analysis was performed based on CRD test with five replications.

In third experiment, single node explants were cultured on N6 medium with 0.8% (w/v) agar supplemented with Kin (0, 1, 2 and 3 mg/l), BAP (0, 1, 2 and 3 mg/l) and TDZ (0, 0.1, 0.2 and 0.3 mg/l). This experiment was conducted as CRD with four replications.

In all experiment, the traits were measured after 30 days. Data analysis was carried out using SPSS 19 software.

3. RESULTS AND DISCUSSION

3.1. MS medium enriched with different concentrations of BAP, Kin and TDZ

First examination-test 1: Analysis of variance showed that the effect of treatments on shoot length, root number, root length and leaf number were significant ($P\leq 0.01$). In this experiment, we obtained very low numbers of shoots (with an average of 2.57 shoots per explants). Therefore, the concentration of TDZ was increased in test 2. The longest shoot (13 cm) and the maximum number of root (10 roots) were obtained from MS medium containing 0.3 mg/l TDZ. In this test, we observed that Kin and TDZ (0.3 mg/l) treatments had the highest effect on root length, but BAP weren't successful on shoot induction and resulted to callus formation and malformed shoot-like structures. Therefore, BAP treatment was removed from test 2 (Table 1, Fig. 1).

Table 1. The effect of different concentrations of Kin, BAP and TDZ on *Stevia rebaudiana* proliferation from single node explants on MS medium.

Phytohormone (mg/l)			Shoot length (cm)	Root number	Root length (cm)	Leaf number
BAP	Kin	TDZ				
-	-	-	2.87 ^c	4 ^{bc}	0.87 ^{cd}	25.5 ^{ab}
1	-	-	1.12 ^c	0 ^c	0 ^d	11.75 ^b
2	-	-	1.37 ^c	0 ^c	0 ^d	9 ^b
3	-	-	0.87 ^c	0 ^c	0 ^d	8.5 ^b
-	1	-	2.37 ^c	2 ^{bc}	2.05 ^{abc}	24.5 ^{ab}
-	2	-	2.37 ^c	3 ^{bc}	2.25 ^{ab}	40 ^{ab}
-	3	-	2.37 ^c	4 ^{bc}	2.95 ^a	54 ^a
-	-	0.1	7.75 ^b	7.5 ^{ab}	1.5 ^{bc}	44 ^{ab}
-	-	0.2	9.25 ^b	7.5 ^{ab}	1.62 ^{bc}	57 ^a
-	-	0.3	13 ^a	10 ^a	2.15 ^{abc}	52 ^a

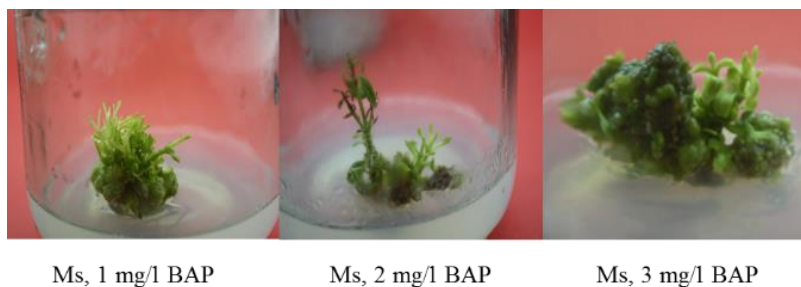


Figure 1. The micropropagated stevia plantlets from single node explants in various concentrations of BAP on MS medium.

First experiment-test 2: Variance analysis revealed significant differences among various concentrations of investigated phytohormones ($P \leq 0.01$) on shoot number, shoot length, root number, root length and leaf number in compared to control (Table 2). In this experiment, the highest shoot number (with an average of 12.6 shoots) and shoot length (with an average of 13.9 cm) were obtained from MS medium containing 0.9 mg/l TDZ (Fig. 2). On the other hand, the lowest shoot number (with an average of 2 shoots) and shoot length (with an average of 3.2 cm) were observed on MS medium without growth regulators (Control). In addition, maximum and minimum number of leaves were produced on MS medium supplemented with 0.9 mg/l TDZ (83 leaves) and control (29 leaves), respectively. Interestingly, root numbers and root length were increased as growth regulators levels increased, and the highest root numbers (14.8 roots) and root length (4.42 cm) were obtained from MS medium supplemented with 0.9 mg/l TDZ (Fig. 3).

Table 2. The effect of different concentrations of Kin and TDZ on *Stevia rebaudiana* proliferation from single node explants on MS medium.

Phytohormone (mg/l)		Shoot length (cm)	Root number	Root length (cm)	Leaf number
Kin	TDZ				
-	-	3.2 ^c	5 ^{bc}	1.1 ^d	29 ^d
1	-	3.7 ^c	2.4 ^c	2.4 ^c	45.2 ^c
2	-	3.8 ^c	3 ^{bc}	3.7 ^{ab}	51 ^c
3	-	4.4 ^c	3.8 ^{bc}	3.8 ^{ab}	69.6 ^{ab}
-	0.3	8.9 ^b	9 ^b	3 ^{bc}	57.2 ^{bc}
-	0.6	10.5 ^b	14.2 ^a	3.9 ^{ab}	69 ^{ab}
-	0.9	13.9 ^a	14.8 ^a	4.42 ^a	83 ^a

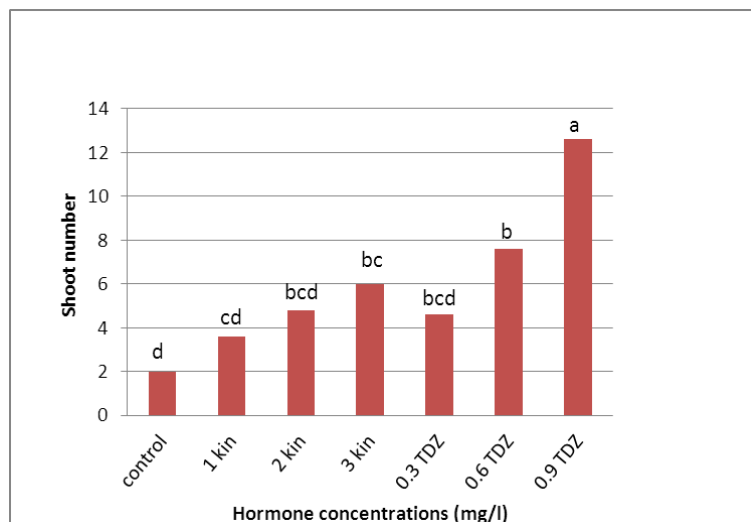


Figure 2. The effect of different concentrations of Kin and TDZ on shoot induction from stevia single node explants incubated on MS medium.

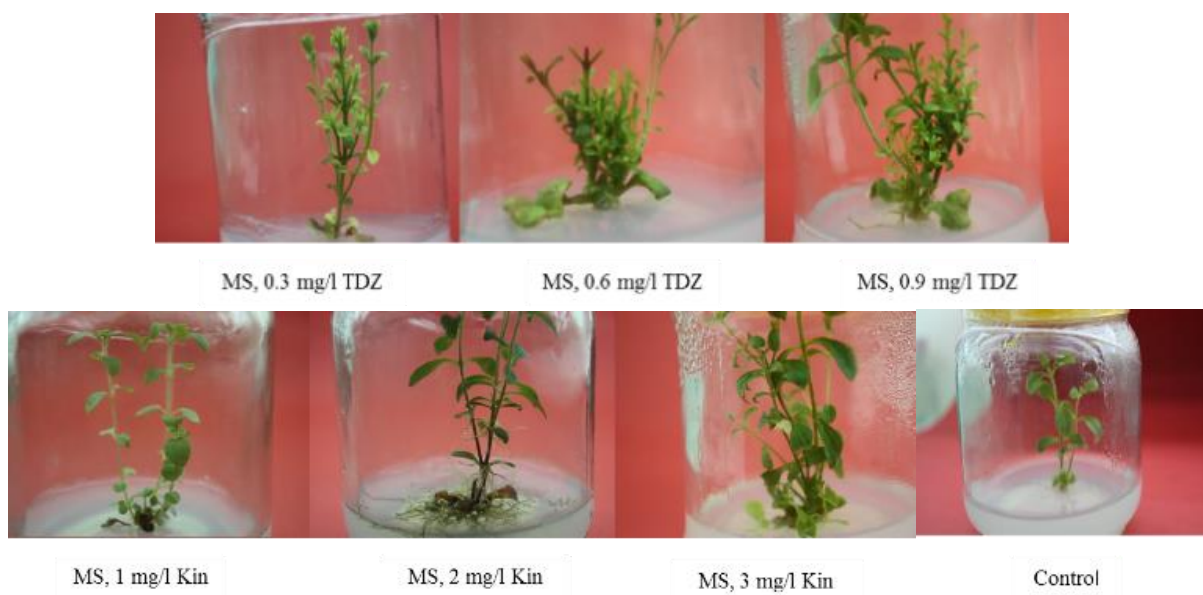


Figure 3. The micropropagated stevia plantlets from single node explants incubated on MS medium enriched with different concentrations of Kin and TDZ.

3.2. B5 medium supplemented with different concentrations of BAP, Kin and TDZ

Second experiment-test 1: The results revealed that various concentrations of investigated phytohormones had significant effect on shoot length, root number, root length and leaf number ($P \leq 0.01$). In this experiment, we obtained 3.3 shoots per explant. The results showed that BAP induced callus production and prevented from shoot and root production. This treatment resulted to malformed shoots and leaves production (Table 3, Fig. 4). Therefore, BAP treatment omitted from test 2. In addition, TDZ concentration enhanced in this test due to inconsiderable effect of added concentrations on desired traits (Table 3, Fig. 4).

Table 3. The effect of different concentrations of BAP, Kin and TDZ on *Stevia rebaudiana* proliferation from single node explants on B5 medium.

Phytohormone (mg/l)			Shoot length (cm)	Root number	Root length (cm)	Leaf number
BAP	Kin	TDZ				
-	-	-	7.37 ^a	43.25 ^a	5.62 ^a	68.5 ^a
1	-	-	1 ^b	0 ^c	0 ^b	14.5 ^b
2	-	-	1.87 ^b	0 ^c	0 ^b	24.66 ^b
3	-	-	3.37 ^b	0 ^c	0 ^b	35.5 ^b
-	1	-	7 ^a	53.75 ^a	5.12 ^a	36.75 ^b
-	2	-	6.5 ^a	13 ^{bc}	3.5 ^a	46.5 ^{ab}
-	3	-	7.78 ^a	6.75 ^{bc}	2.5 ^{ab}	44.5 ^{ab}
-	-	0.1	2.25 ^b	47.5 ^a	3.75 ^a	26 ^b
-	-	0.2	3.62 ^b	24.25 ^b	3.5 ^a	27.5 ^b
-	-	0.3	7.5 ^a	24.5 ^b	4.75 ^a	43 ^{ab}

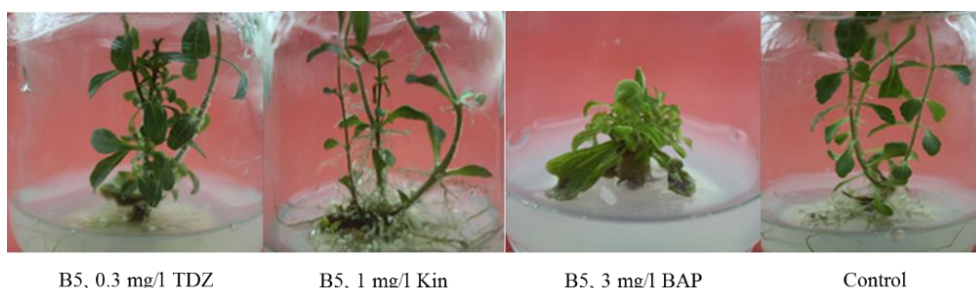


Figure 4. The micropropagated stevia plantlets from single node explants incubated on B5 medium enriched with different concentrations of BAP, Kin and TDZ.

Second experiment-test 2: In this study, the nodal explants were transferred to B5 medium supplemented with Kin and TDZ and observed an enhanced shoot and root proliferation. But, in related to shoot number, the best result obtained from Kin treatment (3 mg/l) was similar to control. In general, the highest number of shoot, shoot length and leaf number were obtained from B5 medium containing 0.9 and 0.6 mg/l TDZ, respectively, but root number was decreased as the TDZ concentration increased. In this test, we were not observed significant difference in root length (Table 4, Fig. 4 & Fig. 5).

Table 4. The effect of different concentrations of Kin and TDZ on *Stevia rebaudiana* proliferation from nodal shoot explants on B5 medium.

Phytohormone (mg/l)		Shoot length (cm)	Root number	Root length (cm)	Leaf number
Kin	TDZ				
-	-	7 ^b	38.6 ^b	4.8 ^a	56.8 ^a
1	-	6.8 ^b	53.8 ^a	4.7 ^a	44.4 ^{bc}
2	-	5.8 ^b	41.6 ^b	3.52 ^{ab}	37 ^c
3	-	7.8 ^b	26.8 ^c	2.92 ^b	41.4 ^{bc}
-	0.3	14.8 ^a	19.8 ^d	4.74 ^a	40 ^{bc}
-	0.6	15.8 ^a	15.6 ^{de}	4.26 ^a	51.4 ^{ab}
-	0.9	16.2 ^a	10.4 ^e	4.2 ^a	58.4 ^a

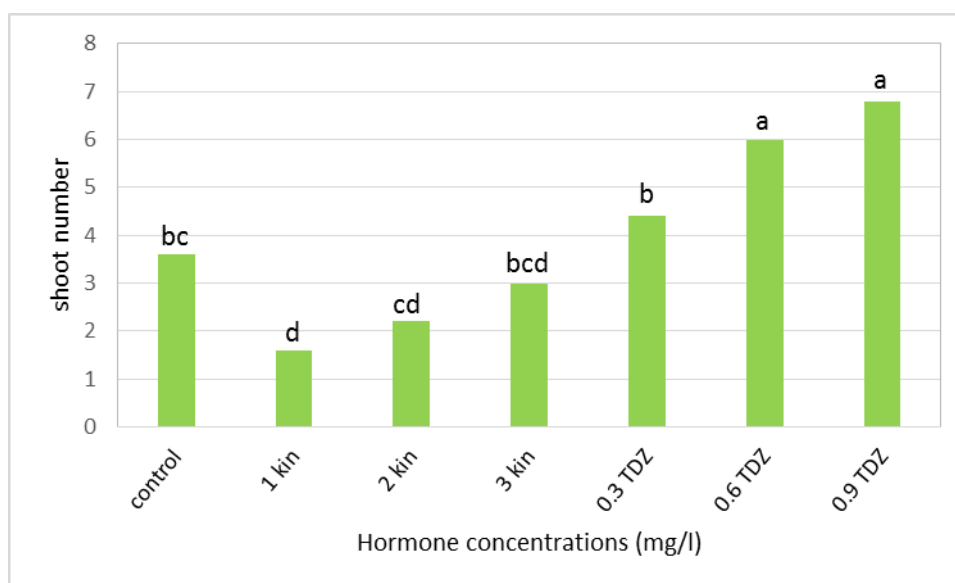


Figure 5. The effect of different concentrations of Kin and TDZ on shoot induction from stevia single node explants incubated on B5 medium.

3.3. N6 medium supplemented with different concentrations of BAP, Kin and TDZ

In N6 Medium each nodal explant produced 1 or 2 axillary shoots. In general, N6 medium caused to callus induction and limited number of malformed shoots production. These plantlets exhibited unsuitable characteristics for micropropagation (Table 5, Fig. 6).

In brief, we observed high shoot number, shoot length and leaf number at high concentration of TDZ (0.9 mg/l) on MS medium. Increasing the leaf number was related to an increase in shoot number, shoot length and node number. In general, TDZ induced more number of shoots than Kin treatments. In MS medium, shoot induction in the lowest concentration of TDZ (0.3 mg/l) didn't show significant difference with highest concentration of Kin (3 mg/l). In a literature, it is reported that TDZ and Kin are the most effective cytokinins in shoot induction of many plants, respectively (Huettelman & Preece 1993). Our results revealed that the type of cytokinin is the most important factor affecting shoot multiplication. This result is in accordance with previous study (Abd Alhady 2011). It is reported that

TDZ stimulates cytokinin autonomy (Capelle et al. 1983). Therefore, the highest number of induced shoots in TDZ treatment could be assigned to this phenomenon. On the other hand, the highest root number and root length were found at 1 mg/l of Kin on B5 medium. It is suggested that chemical composition and salt concentration in the growth medium affect nutrient uptake by plants. B5 medium is composed of low concentration of organic and inorganic components in compared to MS medium. This leads to an increase in nutrient uptake by explant (Agrawal et al. 2003). Therefore, it seems that the high concentration of Kin and TDZ in B5 medium prevent from root induction and its development.

In present research, it was no observed any callus or malformed growth with increasing of Kin and TDZ concentration (Fig 3). In a study, single node explants of stevia genotypes from south-east Rajasthan (India) incubated on MS medium supplemented with Kin (1, 2 and 3 mg/l). MS containing 1, 2 and 3 mg/l Kin induced 2.42, 2.28 and 1.85 shoots and resulted to 6.30, 6.47 and 6.15 cm shoot length, respectively (Jitendra et al. 2012). However, we obtained more number of shoots and shorter shoots with same concentration of Kin. In contrast to their results, we found that shoot number increased with increasing of Kin concentration. This can be attributed to genotype response.

Table 5. The effect of different concentrations of BAP, Kin and TDZ on *Stevia rebaudiana* proliferation from nodal shoot explants on N6 medium.

Phytohormone (mg/l)			Shoot length (cm)	Root number	Root length (cm)
BAP	Kin	TDZ			
-	-	-	0.37 ^b	14 ^a	3.3 ^a
1	-	-	1.25 ^{ab}	0 ^c	0 ^c
2	-	-	1.75 ^a	0 ^c	0 ^c
3	-	-	0.43 ^b	0 ^c	0 ^c
-	1	-	0.42 ^b	1.75 ^c	2.12 ^{ab}
-	2	-	0.42 ^b	1 ^c	1.5 ^{bc}
-	3	-	0.75 ^b	1.75 ^c	1.25 ^{bc}
-	-	0.1	0.5 ^b	2.5 ^c	1.7 ^{bc}
-	-	0.2	0.45 ^b	2.5 ^c	1.37 ^{bc}
-	-	0.3	0.55 ^b	6.5 ^b	1.52 ^{bc}

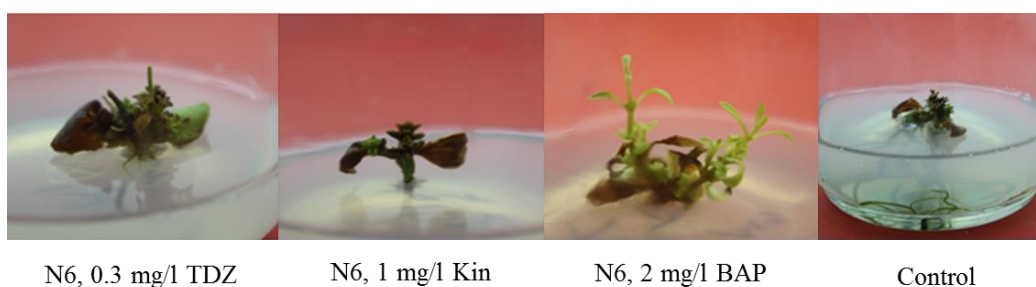


Figure 6. The micropropagated stevia plantlets from single node explants incubated on N6 medium enriched with different concentrations of BAP, Kin and TDZ.

4. CONCLUSIONS

In conclusion, we found that plant growth regulators, their concentration and genotypes have remarkable impact on proliferation of stevia. In present study, the effect of Kin, BAP and TDZ and different basal medium on micropropagation of stevia single node explants were studied and major conclusion are as follows:

1. The highest number of shoots and leaves obtained from MS medium containing 0.9 mg/l TDZ.
2. The longest shoots were related to B5 medium supplemented with 0.9 mg/l TDZ.
3. Maximum number of roots was observed on B5 medium containing 1 mg/l Kin.
4. Single node cuttings planted on N6 medium exhibited malformed structures and unsuitable characteristics for micropropagation.

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REFERENCES

- Abd Alhady, M.R.A., 2011. Micropropagation of *Stevia rebaudiana* Bertoni.: a new sweetening crop in Egypt. *Global Journal of Biotechnology & Biochemistry*, 6(4), pp.178–182.
- Agrawal, S., Chandra, N. & Kothari, S.L., 2003. Plant regeneration in tissue cultures of pepper (*Capsicum annuum* L. cv. Mathania). *Plant Cell, Tissue and Organ Culture*, 16, pp.47–55.
- Ahmed, M.B. et al., 2007. An efficient method for *in vitro* clonal propagation of a newly introduced sweetener plant (*Stevia rebaudiana* Bertoni.) in Bangladesh. *American-Eurasian Journal of Scientific Research*, 2(2), pp.121–125.
- Anbazhagan, M. et al., 2010. *In vitro* production of *Stevia rebaudiana* Bertoni. *Emirates Journal of Food and Agriculture*, 22(3), pp.216–222.
- Banerjee, M. & Sarkar, P., 2008. *In vitro* callusing in *Stevia rebaudiana* Bertoni using cyanobacterial media-a novel approach to tissue culture. *International Journal of Integrative Biology*, 3(3), pp.163–168.
- Ben-Jaacov, J. et al., 1991. Vegetative propagation of *Albertya magna* by tissue culture and grafting. *HortScience*, 26, p.74.
- Capelle, S.C. et al., 1983. Effects of thidiazuron on cytokinin autonomy and the metabolism of N6-(Y2-Isopentyl) [8-14c] Adeno- sine in callus tissues of *Phaseolus lunatus* L. *Plant Physiology*, 73(3), pp.796–802.
- Das, A., Gantait, S. & Mandal, N., 2011. Micropropagation of an elite medicinal plant: *Stevia rebaudiana* Bert. *International Journal of Agricultural Research*, 6, pp.40–48.
- Fatima, A. & Khan, S.J., 2010. Som factors affecting the *in vitro* growth of *Stevia rebaudiana* Bertoni. *Iranian Journal of Plant Physiology*, 1(2), pp.61–68.
- Gupta, P., Sharma, S. & Saxena, S., 2010. Callusing in *Stevia rebaudiana* (natural sweetener) for steviol glycoside production. *International Journal of Agricultural and Biological Science*, 1(1), pp.30–34.
- Huetteman, C.A. & Preece, J.E., 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture*, 33(2), pp.105–119.

- Jitendra, M. et al., 2012. Micropropagation of an anti diabetic plant-*Stevia rebaudiana* Bertoni, (natural sweetener) in Hadoti region of south-east Rajasthan, India. *ISCA Journal of Biological Sciences*, 1(3), pp.37–42.
- Miyagawa, H. et al., 1986. Studies on the tissue culture of *Stevia rebaudiana* and its components. II. Induction of shoot primordial. *Planta Medica*, 52, pp.321–323.
- Murashige, T. & Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3), pp.473–497.
- Overvoorde, P., Fukaki, H. & Beeckman, T., 2010. Auxin control of root development. *Cold Spring Harbor Perspectives Biology*, 2(6), p.a001537.
- Richman, A.S. et al., 1999. Diterpene synthesis in *Stevia rebaudiana* Bert. recruitment and up-regulation of key enzymes from the gibberellin biosynthetic pathway. *Plant Journal*, 19, pp.411–421.
- Starr, C. et al., 2012. *Biology: the unity and diversity of life* 13th editi., Brooks Cole.
- Starratt, A.N. et al., 2002. Rebaudioside F, a diterpene glycoside from *Stevia rebaudiana*. *Phytochemistry*, 59, pp.367–370.
- Yang, Y.W., Hsing, Y.I. & Chang, W.C., 1981. Clonal propagation of *Stevia rebaudiana* Bertoni through axillary shoot proliferation *in vitro*. *Botanical Bulletin- Academia Sinica*, 22, pp.57–62.