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Full Length Research Paper

Study of the effect of physical state of medium and different concentrations of sucrose, ferric ethylenediamine- tetraacetic acid (FeEDTA) and $CuSO_4$ in enhancing the micropropagation system of *Stevia rebaudiana*

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Stevia rebaudiana **is a sweet herb native to Paraguay and Brazil. Its leaves are well known for containing sweet glycosides which are intensely sweet compounds with zero caloric value. A comparison of effect of state of medium on the *in vitro* growth of *S. rebaudiana* was studied under controlled conditions. Different concentrations of benzyl amino purine (BAP) and kinetin (Kin) (1, 2 and 3 mg/L) were used for the purpose. Explants inoculated in liquid media placed on orbital |shaker showed the best results for the vigorous growth of the plant in all the hormone treatments. In all the hormone treatments, shaking media gave the maximum values of number of multiple shoots as well as shoot length. Shaking cultures also showed response in minimum days of inoculation as compared to solid and static liquid cultures of honey leaf, *S. rebaudiana*. Medium conditions were also optimized for the maximum micropropagation potential of the *S. rebaudiana* explants in shaking medium by adding different concentrations of Fe- EDTA, $CuSO_4$ and sucrose. Optimal concentrations were determined as 60 g/L sucrose, 9 ml/L Fe-EDTA and 27.5 µg/L $CuSO_4$.**

Key words: Benzyl amino purine (BAP), kin, liquid medium, *Stevia rebaudiana*, $CuSO_4$, ferric ethylenediamine-tetraacetic acid (FeEDTA), sucrose.

INTRODUCTION

Stevia rebaudiana, native to South America, is considered as a valuable medicinal plant due to the presence of sweet glycosides in its leaves. Stevioside and rebaudioside A are the main glycosides responsible for its sweet taste. Other glycosides present are- steviol bioside, rebaudioside B, C, D E, F and dulcoside A (Geuns, 2003; Kennelly, 2002; Soejarto, 2002). Stevioside is 110 to 300 times sweeter than sucrose (Megejii et al., 2003; Uddin et al., 2006) but has no caloric value (Dacome et al., 2005; Cardello et al., 1999). Primary use of stevioside is to increase the taste of the products by simply increasing the sweetness and decreasing the health hazards of the product. It is also added to the pills, capsules and syrups to increase their palatability (Bhise and Salukhe, 2009; Dacome et al., 2005). World health organization (WHO) has also recognized that stevioside is not genotoxic and daily intake of Stevioside as much as 0 to 2 mg/Kg of body weight is safe (Benford et al., 2006). Seed germination of this precious medicinal plant is very poor (Duke, 1994). Another novel approach for multiplication of *S. rebaudiana* is tissue culture (Seema, 2010; Mitra and Pal, 2007; Patil et al., 1996; Ali et al, 2010). A number of

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Abbreviations: BAP, Benzyl amino purine; **Kin,** kinetin; **MS,** Murashige and Skoog.

Table 1. Effect of state of medium on number of multiple shoots of *S. rebaudiana.*

MS medium (mg/L)	Medium			LSD
	Solid	Liquid	Agitated liquid	
BAP 1	3.46±0.07^{c}	10.34±0.35^{b}	21.11±0.56^{a}	1.07
BAP 2	2.16±0.08^{c}	14.3±0.17^{b}	29.2±0.63^{a}	1.43
BAP 2	2.14±0.12^{c}	16.54±0.23^{b}	30.78±0.65^{a}	1.15
Kin 1	2.98±0.06^{c}	5.06±0.08^{b}	16.82±0.22^{a}	0.51
Kin 2	3.18±0.12^{c}	6.58±0.14^{b}	11.98±0.14^{a}	0.45
Kin 3	4.04±0.11^{c}	8.88±0.26^{b}	14.24±0.21^{a}	0.85

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range tests. The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

research reports and papers are available on solid culture systems (Patel and Shah, 2009; Ahmed et al., 2007; Rafiq et al., 2006) as well as for liquid culture systems (Kalpana et al., 2009) for the micropropagation and mass production of the *S. rebaudiana*. But no comparison of these systems is still available. It is a well known fact that establishment of the liquid cultures makes the growth faster (Ascough and Fennel, 2004). It also removes the chances of impurities from the medium due to agar or any other solidifying medium and lessens the cost of the medium due to the removal of the solidifying agent (Ziv and Halvey, 1983). While shaking/agitating of the cultures on the shakers increase the availability of the nutrients and phytohormones to the explants. So in this research work an attempt is made to clarify the difference between growths in solid cultures, static liquid cultures and shaking liquid cultures. Meanwhile nutrients' concentrations for agitating medium are also optimized to develop a more suitable protocol for the mass propagation of the *S. rebaudiana*.

MATERIALS AND METHODS

Shoot tips of *S. rebaudiana* plant were used as explants for the research work. These explants were taken from the green house grown plants from Lahore College for Women University, Lahore, Pakistan. Shoot tips were first washed with the running tap water. In the next step all the shoot tips were treated with household detergent (Surf Excel) to remove the dust particles from the surface of the explants, following the washing with running tap water to remove the detergent. The explants were sterilized by dipping in 20% sodium hypochlorite solution for 15 min. After 15 min explants were washed with autoclaved water to remove the traces of the sodium hypochlorite under the laminar airflow cabinet. The sterilized shoot tips were inoculated in different media. After achieving sterilization the shoot tips were inoculated in different media (1. Solidified with phytagel; 2. Liquid medium in static conditions (jars), and 3. Liquid medium in agitated condition) containing different concentrations of Kin and BAP. A set of treatments were applied to all hormones doses of the media. Treatment 1 contained solidified media with phytagel (1.5 g/L). Treatment 2 contained liquid medium using sterilized cotton as supporting medium. Treatment 3 included liquid media in flasks on shaker, shaking at speed of 80 rounds per minute.

After this, an experiment was designed to optimize the media formulations for the liquid shaking medium. For this purpose 7 different concentrations of BAP and kinetin (1, 2, 3, 4, 5, 10 and 15) were used. Data were recorded for the number of shoots and average shoot lengths and best selected media were then further modified with different treatments of sucrose (Table 3), Fe-EDTA (Table 4) and $CuSO_4$ (Table 5). The pH of media was set at 5.4 to 5.7 for all experiments. Each time medium was sterilized by autoclaving at 121°C and 15 Ibs/Inch^2 pressure for 20 min. Cultures were maintained in culture room under controlled conditions with fluorescent light having 2500 flux light intensity, temperature range of 22 ±2°C with 16 h light and 8 h dark period in every 24 h cycle. The data were recorded for number of shoots per explants and for average shoot length. The experimental design was completely randomized with five replicates for each medium state. Analysis of variance (ANOVA) depicting significance among means was calculated by applying Duncan's new multiple range test at 5% level of significance, using Costat V.63: Statistical Software (Cohort Software, Berkley, California).

RESULTS

Shoot tips of *S. rebaudiana* were inoculated in three different states of Murashige and Skoog (1962), (MS) medium; (1) solidified with phytagel, (2) liquid medium in static conditions (jars), and (3) liquid medium in agitated condition containing different concentrations of Kin and BAP. Results were estimated by studying the parameters like number of branches and shoot length. Table 1 depicts the fact that treatment three showed the maximum number of shoots in all the phytohormones concentrations (Figure 2A). Its value ranged from 2.14±0.12 shoots per explants on average in solid MS + BAP 3 mg/L (Figure 2B) to 30.78±0.65 shoots per explant in agitated liquid medium that is, MS+BAP 3 mg/L (Figure 2c). Table 2 also advocates the results of Table 1 as using all the hormone treatments, the shoot length gradually increased in order from solid, static liquid to agitating liquid medium. Maximum shoot length was obtained in agitating liquid medium that is, MS medium containing 1 mg/L BAP (Figure 2D and 2E). Figure 1 depicts the comparison of BAP and Kin in agitated liquid

Table 2. Effect of state of medium on shoot length (cm) of *S. rebaudiana.*

MS medium (mg/l)	Medium			LSD
	Solid	Liquid	Agitated liquid	
BAP 1	2.46±0.11^{c}	3.36±0.06^{b}	7.16±0.16^{a}	0.51
BAP 2	3.92±0.09^{c}	4.94±0.11^{b}	5.2±0.19^{a}	0.52
BAP 2	2.08±0.03^{c}	2.94±0.06^{b}	3.74±0.08^{a}	0.3
Kin 1	3.1±0.06^{c}	4.08±0.12^{b}	5.1±0.10^{a}	0.41
Kin 2	4.2±0.12^{c}	4.86±0.19^{b}	6.4±0.15^{a}	0.57
Kin 3	4.16±0.15^{c}	4.84±0.20^{b}	6.52±0.09^{a}	0.63

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range tests. The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

Table 3. Optimization of the sucrose concentration in the medium for shaking liquid cultures of *S. rebaudiana.*

S/N	Sucrose		BAP 1 mg/L		BAP 3 mg/L	
	Age in the medium (%)	Original concentration (g/L)	Number of shoots	Average shoot length (cm)	Number of shoots	Average shoot length (cm)
1	1	30.3	18.54±0.58^{j}	8.82±0.12^{ef}	40.04±0.26^{g}	3.74±0.15^{hi}
2	2	30.6	18.16±0.29^{k}	9.24±0.21^{cd}	39.96±0.18^{g}	3.86±0.19^{gh}
3	5	31.5	18.42±0.23^{jk}	9.08±0.24^{de}	40.72±0.48^{f}	3.52±0.07^{ij}
4	10	33.0	19.84±0.14^{i}	9.42±0.28^{bc}	41.02±0.60^{ef}	4.10±0.27^{fg}
5	15	34.5	23.16±0.29^{f}	9.48±0.12^{bc}	41.22±0.41^{ef}	4.28±0.13^{def}
6	20	36.0	27.74±0.04^{e}	9.74±0.10^{a}	41.50±0.27^{de}	4.44±0.24^{cde}
7	40	42.0	28.94±0.18^{d}	9.78±0.16^{a}	41.92±0.29^{cd}	4.56±0.05^{bcd}
8	60	48.0	31.36±0.19^{b}	9.86±0.19^{a}	42.32±0.30^{c}	4.74±0.24^{abc}
9	80	54.0	31.50±0.28^{b}	9.8±0.11^{a}	43.16±0.26^{b}	4.80±0.17^{ab}
10	100	60.0	34.3±0.27^{a}	9.64±0.13^{ab}	45.9±0.49^{a}	4.96±0.15^{a}
11	120	66.0	29.34±0.27^{c}	9.44±0.30^{bc}	42.3±0.24^{c}	4.64±0.14^{bc}
12	140	72.0	21.32±0.26^{g}	9.22±0.17^{cd}	42.4±0.22^{c}	4.14±0.24^{efg}
13	160	78.0	20.94±0.11^{h}	8.92±0.07^{ef}	42.0±0.29^{c}	4.02±0.39^{fgh}
14	180	84.0	14.12±0.2^{l}	8.90±0.19^{ef}	31.8±0.19^{h}	3.78±0.20^{ih}
15	200	90.0	10.28±0.23^{m}	8.76±0.08^{f}	26.2±0.21^{i}	3.38±0.0^{j}
LSD			0.34	0.25	0.49	0.29

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range test. The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

liquid medium in terms of shoot length and number of shoots per culture vial. It is clear from the results that agitated liquid medium that is, MS medium containing 1 mg/L BAP proved to be the best medium in terms of shoot length and agitated liquid MS medium containing 3 mg/L BAP in terms of number of shoots per culture vial. Table 3 shows the effects of sucrose concentrations on micropropagation of *S. rebaudiana.* When concentration of sucrose was increased up to 200% of the original concentrations, it did not affect shoot length. But much higher concentrations of sucrose decreased the average number of shoots per culture vial. When concentration of sucrose in the medium was increased to 100% that is, 60 g/L (Table 3) and maximum number of shoots per culture vial was obtained in agitating MS medium supplemented with 3 mg/L BAP.

Table 4 explains the role of Fe-EDTA on *in vitro* growth of *S. rebaudiana* plant. Results clearly depicts it also did not affect the shoot length but number of shoots per explants increased gradually by increasing the concentration of Fe-EDTA in agitated MS medium up to an optimum concentration of Fe-EDTA that is, 80% of the original MS medium or 9 ml/l of the MS medium (Figure 2F). Data presented in Table 5 depicts the effect of the $CuSO_4$ on micropropagation of the *S. rebaudiana* in agitated liquid MS medium supplemented with optimized

Table 4. Optimization of the Fe-EDTA concentration in the medium for shaking liquid cultures of *S. rebaudiana.*

S/N	Fe-EDTA		BAP 1 mg/L		BAP 3 mg/L	
	Age in the medium (%)	Original concentration (ml/L)	Number of shoots	Average shoot length (cm)	Number of shoots	Average shoot length (cm)
1	1	5.05	20.42±0.58^{g}	9.00^{d}±0.11	39.48±0.41^{g}	4.10±0.4 1^{c}
2	5	5.25	22.96±0.26^{e}	9.18^{cd}±0.18	40.18±0.34^{f}	4.28±0.17^{c}
3	10	5.50	23.46±0.38^{e}	9.26^{cd}±0.18	40.28±0.53^{f}	4.32±0.17^{bc}
4	20	6.00	25.1±0.25^{d}	9.32^{c}±0.20	41.12±0.23^{e}	4.38±0.24^{bc}
5	40	7.00	31.02±0.04^{c}	9.42^{bc}±0.35	41.32±0.49^{e}	4.42±0.07^{bc}
6	60	8.00	34.48±0.32^{b}	9.48^{bc}±0.23	43.26±0.44^{d}	4.52±0.28^{abc}
7	80	9.00	36.84±0.10^{a}	9.66^{ab}±0.17	45.46±0.55^{c}	4.76±0.15^{ab}
8	100	10.0	31.16±0.53^{c}	9.72^{ab}±0.19	47.12±0.35^{b}	4.86±0.15^{a}
9	120	11.0	22.14±0.26^{f}	9.83^{a}±0.13	49.72±0.19^{a}	4.94±0.22
10	140	12.0	20.30±0.38^{g}	9.96^{a}±0.14	47.62±0.37 ^{b}	4.96±0.29^{a}
11	160	13.0	14.9±0.66^{h}	9.80^{a}±0.19	37.76±0.7 ^{h}	4.86±0.61^{a}
	LSD		0.53	0.28	0.62	0.42

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range tests. The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

Table 5. Optimization of the $CuSO_4$ concentration in the medium for shaking liquid cultures of *S. rebaudiana.*

S/N	$CuSO_4$		BAP 1 mg/L		BAP 3 mg/L	
	Age in the medium (%)	Original concentration (µg/L)	Number of shoots	Average shoot length	Number of shoots	Average shoot length
1	1	25.25	20.66±0.39^{b}	3.24±0.10^{a}	40.50±0.46^{a}	3.96±0.26^{a}
2	5	26.25	21.06±0.51^{b}	3.14±0.11^{a}	42.26±0.25^{b}	3.2±0.19^{b}
3	10	27.50	25.50±0.32^{a}	3.08±0.23^{a}	45.36±0.42^{a}	2.54±0.22^{c}
4	20	30.00	19.80±0.35^{c}	3.02±0.27^{a}	39.62±0.29^{d}	1.76±0.38^{d}
5	30	32.50	14.34±0.31^{d}	2.98±0.21^{a}	22.28±0.23^{e}	1.50±0.19^{de}
6	40	35.00	10.8±0.43^{e}	2.48±0.29^{b}	14.28±0.73^{f}	1.22±0.02^{e}
	LSD		0.67	0.33	0.61	0.36

Means followed by different letters in the same column differ significantly at p=0.05 according to Duncan's new multiple range tests. The results were calculated from three replicated experiments for each treatment, each with 10 explants per treatment.

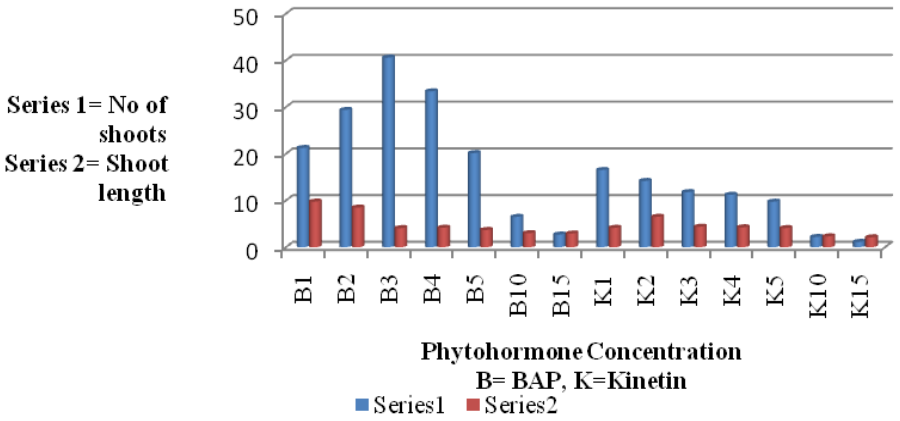

Figure 1. Effect of type of cytokinin on shoot growth of *S. rebaudiana* in agitating liquid medium.

Figure 2. Slides A to F, showing effect of different factors on micropropagation of *S. rebaudiana* in solid and agitating liquid medium; **A**: Solid cultures of MS + BAP 1 mg/L; **B**. Liquid cultures of *S. rebaudiana* without agitation in MS+BAP 3 mg/L; **C.** Liquid cultures of *S. rebaudiana* with agitation in MS + BAP 3 mg/L; **D**. Liquid cultures of *S. rebaudiana* with agitation in MS + BAP 1 mg/L; **E**. Liquid cultures of *S. rebaudiana* without agitation in MS+ BAP 1mg/L; **F**. Liquid cultures of *S. rebaudiana* with agitation in MS + BAP 3 mg/L+ 9 ml/L Fe-EDTA.

concentrations of the phytoregulators. It is obvious from the results that an increase in the concentration of the $CuSO_4$ in the MS medium had a positive effect on the number of the shoots per explants of *S. rebaudiana* but a negative effect on the shoot length of the crop (Table 5).

DISCUSSION

Our results are in accordance with the findings of Kalpana et al. (2009) who reported that liquid shoot cultures gave better results for the micropropagation of the *S. rebaudiana* in different combinations of BAP and indole acetic acid (IAA). Scientists working with the other plants also reported the same facts in different times. Kim et al. (2003) found better shoot length and rhizome weight for garlic in liquid cultures as compared to the solid cultures. Micropropagation of tea plant was also noticed to be better in liquid cultures (Sandal et al., 2001). Avila et al. (1996) observed better tuber and shoot weight of potato in liquid cultures. Jackson et al. (1991) reported that agitation of liquid medium increases the availability of the nutrients to all parts of the explants. It also reduces the depletion zones around the explants which are usually formed in solid media due to utilization by actively growing explants tissues. These cultures with agitation also increase the aeration of the explants (Whitehouse et al., 2002).

Gruel and Gulsen (1998) used 6% sucrose for the optimum shooting in the almond plant, but they find the inverse effect to present research on the shoot length of the plants, further more Khan et al. (2006) reported that 6% sucrose in the medium increased the rooting capability of the sugarcane plant. Dahab et al. (2005) used 30 g/L sucrose for the maximum efficiency of the medium for the micropropagation of the Ruscus sp. $CuSO_4$ increased the number of buds or branches per plant. Findings of many other scientists also reaffirm our

results. Dahleen (1995) used higher concentration of $CuSO_4$ for Barley, Tahiliani and Kothari (2004) for wheat, Joshi and Kothari (2007) for capsicum, Jain et al. (2009) for Stevia, and Kaul-Khurana et al. (2010) for *Jatropha curcus* in MS medium to enhance the number of branches per culture vial in different plants to different extent. But still no research work was found in relation to the effect of the Fe-EDTA on the shoot multiplication capability of the *in vitro* grown plants.

Conclusion

Agitating liquid medium has been found to be preferable over both solid and liquid medium for commercial micropropagation of the *S. rebaudiana.* These findings also confirmed that agitated liquid MS medium containing 3 mg/L BAP can be used for the massive micropropagation of *S. rebaudiana* and agitating liquid MS medium containing 1 mg/L BAP can be used along with rooting medium for the elongation of the twigs of the *S. rebaudiana* to be settled for the hardening stage. Best medium formulated for the commercial and massive micropropagation of the *S. rebaudiana* was MS medium supplemented with 3 mg/L BAP, 60 g/L sucrose, 9 ml/L Fe-EDTA and 27.5 µg/L $CuSO_4$.

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