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Direct Shoot Bud Proliferation Protocol from *Stevia rebaudiana* Leaf Culture for Healthy Biomass Production

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AN EFFICIENT and simple regeneration protocol using leaf explants was described on MS medium in the presence of various plant growth regulators. After four weeks of culture, the highest induction of adventitious shoot buds from leaf culture (90%) was achieved on MS medium with 1.0mg/L BA. The largest number of shoot buds (3.02) per leaf explant with no callus formation was achieved on MS medium with 2.0mg/L BA. The best combination for the induction of numerous shoot buds was found to be TDZ + BA + IBA (0.5, 1.0, and 0.5mg/L, respectively), which presented 10.4 shoots per explant with 3.15cm in length. For *in vitro* root formation stage, Healthy shoots were collected and cultivated on a half-strength MS with various concentrations of NAA and IAA alone or in combination with 2,4-D or IBA. Furthermore, the highest roots (8.17) per shoot with root formation (100 %) was observed using a half-strength MS medium in the presence of 2.0mg/L IAA. Therefore, healthy *in vitro* rooted plantlets were acclimatized successfully after four weeks in the greenhouse.

Keywords: *In vitro* rooting, Leaf explant, Plant growth regulators, Plant regeneration, *Stevia rebaudiana*.

Introduction

Diabetes is a common disease that affects people all over the world, including Egyptians. It is expected to impact 57 million people by 2025, according to estimates. Nowadays, a few pharmaceutical firms and people are turning to herbal remedies that is which is thought to be less toxic and free of side effects than synthetic medicine (Attaya, 2017). Candyleaf *Stevia rebaudiana* Bertoni (Asteraceae family), a medicinal and valuable plant containing sweetener compounds in their leaves, that are found to be 100-300 times sweeter than sucrose in its leaves (Ahmad et al., 2011). Stevia has been used as a natural sweetener in a broad range of products in the food and soft drink industry for decades since its leaves contain stevioside and rebaudioside (Chaturvedula & Meneni, 2018). These compounds are being used for the treatment of diabetes because they are non-toxic, low-calorie, and non-mutagenic (Aman et al., 2013). Stevioside is very important for hypertension, depression, fatigue, and infections (Dyrskog et al., 2005). The stevioside also used in processed foods, bakings, coffee, tea, beverages, cold drinks, and fruit juices (Deshmukh & Ade, 2012). The stevioside also important as anti hypersensitive properties, anti cancerous, anti-pathogenic, antioxidant activities (Starratt et al., 2002).

Stevia seeds are small, lose viability during storage and have a poor germination rate. Moreover, propagation by seeds leads to great variability in sweetening levels and composition regarding it prevents the development of homogeneous populations (Nakamura & Tamura, 1985; Mitra & Pal, 2007). Vegetative propagation through stem cuttings require more input stock and are much more time consuming (Debnath et al., 2006; Mitra & Pal, 2007). *In vitro* propagation technique is an attractive option in a short time for large scale production (Pande & Gupta, 2013; Ramirez-Mosqueda & Iglesias-Andreu, 2015; Attaya, 2017). The growing demand for high-

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quality materials has highlighted the importance of plant tissue culture technology for rapid and mass multiplication. As a result, in vivo and in vitro nodal explants were used to demonstrate high frequency Stevia mass multiplication (Singh et al., 2017).

Using leaf explants has a greater multiplication potential because it may induce a multitude of new shoots, depending on the regeneration capacity. The downside of the use of leaf explants is that a longer or shorter episode of callus formation precedes the initiation of a shoot (Attaya et al., 2012). Shoot buds (4.93) with 93% response were obtained directly from the upper epidermis of immature Stevia leaf explants on MS medium in the presence of (8.88µM) BA, and kinetin ranging (4.65 to 6.98µM) (Sreedhar et al., 2008). Moreover, MS medium in the presence of IBA ranging (4.92 to 7.38µM) was found to be suitable for shoot bud elongation. Leaf explants were maintained on MS medium with various concentrations of BA, Kn, and IAA individual or in combination forms for shoot inductions (Anbazhagan et al., 2010), and the optimum values were observed from MS medium combined with BA+ IAA at concentrations of (1.0 and 0.5mg/L). (Preethi et al., 2011) developed a direct shoot regeneration protocol from *Stevia rebaudiana* leaf explants. The highest shoots number (10.4) was achieved on MS medium in the presence of BA+ Kn+ IAA at the amounts of (1.0, 0.5, and 0.1mg/L, respectively), whereas in in vitro derived leaf explants, the highest shoots number (28.7) was obtained on MS medium in the presence of BA+ Kn+ NAA at (2.0, 0.5, and 0.1mg/L, respectively). Ahmad et al. (2020) investigated the effect of engineered nanoparticles ZnO and CuO on in vitro root formation development of Candyleaf on MS medium, the rooting percentage was 91% and 94% by using 2mg/L of ZnO or CuO, respectively.

The study present an efficient protocol for high-frequency direct regeneration of *Stevia rebaudiana* plantlets from leaf explants that shows no or limited callus induction for mass propagation and genetic improvement.

Materials and Methods

Sterilization of explants and plant material

Sugar Crops Research Institute, Agricultural Research Center, Ministry of Agriculture, Egypt collected *Stevia rebaudiana* seeds var. spanti. The seeds were germinated and maintained in the greenhouse of Plant Science Department, McGill University, Canada. Stevia plants were raised in pots containing garden soil and farmyard manure (1:1) under greenhouse condition. Leaf explants were collected from potted plants and processed for aseptic culture. At plant tissue culture laboratory, leaf explants were cleaned thoroughly using flowing tap water and submerged in a flask containing a few drops of Tween-20 in tap water, followed by Hand shacking for 5min., and then rinsing in tap water to extract the soap. Explants were surface sterilised with 70% (v/v) ethanol for 30sec. and then with 0.15 percent mercuric chloride ($HgCl_2$) solution for 1min.under a laminar airflow hood before being washed three times with sterile distilled water to eliminate all traces of $HgCl_2$.

Culturing medium and conditions

The sterilized leaf explants were cut into small pieces (1.0-1.5cm diameter) and cultured in contact with MS medium (Murashige & Skoog, 1962) supplemented with 30g L^{-1} sucrose and 7g L^{-1} of agar. Until gelling with agar and autoclaving at 121°C and 1.1kg cm^{-2} for 20min, the pH was set to 5.6-5.7. During the in vitro establishment stage, 1.0mg/L of BA was added to the MS medium. Then, cytokinins as TDZ and BA with different concentrations (0, 0.5, 1.0 and 2.0mg/L) were added to MS medium to investigate direct adventitious shoot bud induction on *Stevia rebaudiana* leaf discs. Moreover, to study the effect of plant growth regulators on direct adventitious shoot bud regeneration, different concentrations (0.1, 0.5 and 1.0mg/L) and combinations from plant growth regulators (TDZ, BA, IAA and NAA) were added to MS medium. The treatment was contained four replicates, which five leaf disc explants were cultured as a replicate. In an air-conditioned culture room, the cultures were held at 25±2°C with relative humidity of 70±5% and a photoperiod of 16 hour per day supported by cool white fluorescent lamps (light intensity 2000 Lux.).

Root formation

In vitro shoots (1-2cm long) regenerated from leaf explants were cultured on half-strength MS medium supplemented with 7g L^{-1} agar and 30g L^{-1} sucrose. To monitor the initiation and quality of adventitious roots on the regenerated shoots, Auxins as IAA and NAA with different concentrations (0, 0.1, 0.5, 1.0 and 2.0mg/L) were

added to MS medium. Moreover, using 0.5mg/L IAA in combination with different concentrations (0.1, 0.5 and 1.0mg/L) of NAA, IBA and 2,4-D. The treatment was contained four replicates, which five shoots were cultured as a replicate. The cultures were held in the same air-conditioned culture room with the same conditions.

Acclimatization stage

After 6 weeks, the well-developed stable *in vitro* rooted plantlets were carefully removed from the medium and washed with sterilised distilled water before being transplanted ex vitro. The plantlets were then placed in 100mL pots containing an organic soil and sand mixture (1:1 ratio). Then, to retain humidity, it was put in a plastic tunnel and wetted with tap water before being covered with clear plastic bags. During the three weeks, the plants were watered three times. The developed plants were then transplanted into black polyethylene bags with garden soil and farmyard manure for continued growth.

Analytical statistics

Duncan's multiple range test (DMRT) was used to measure the statistical difference between the treatment means at the 0.05 level using SPSS (version 17), and the results were expressed as the mean ± SE. An analysis of variance (ANOVA) was also performed on the data.

Results and Discussion

Direct adventitious shoot bud induction from leaf explants

Data presented in Table, 1 cleared statistically significant variations between treatments at 0.5 level after 4-weeks of culture. The optimum number of adventitious shoot buds from leaf culture (3.02) with 85% shoot induction was occurred using 2.0mg/L BA without callus formation. While the optimum shoot induction from leaf culture (90%) with (2.27) adventitious shoot buds/explant and shoot length (2.50cm) were observed by using 1.0mg/L BA. The results showed that BA was more effective than TDZ. These results seems to be in harmony with those of Ahmad et al. (2011), Atalay et al. (2011), Aman et al. (2013). Whereas, the tallest shoots were occurred using 0.5mg/L TDZ recording 3.32cm shoot length with 2.20 shoots/explant. BA was proved the most favorable and suitable cytokinin for shoot bud multiplication that gave 80-90% of shoot induction while TDZ was proved the most efficient cytokinin for shoot elongation, and gave 60-70% of shoot induction. Thus, increasing the concentration of BA to more than 0.5mg/L leads to increased shoot number. While increasing TDZ concentration more than 0.5mg/L reduced number of shoots and shoot elongation but increase callus formation from zero to 10mm in diameter. These results seems to be in harmony with those of Singh et al. (2017), Keshvari et al. (2018).

Regarding previous published works of other research groups, of those two cytokinins tested, and BA revealed to become more favorable and efficient for shoot bud growth from leaf explants (Sivaram & Mukundan, 2003; Preethi et al., 2011). There are several reasons for preferred use of BA; it can be autoclaved without losing its activity and slow degradation. In various plant species, similar results have been presented to study the influence of BA on the induction of multiple shoot buds on *Quercus euboica* (Kartsonas & Papafotiou, 2007), *Ulmus parvifolia* (Thakur & Karnosky, 2007) and on *Sacostemma brevistigma* (Thomas & Shankar, 2009).

TABLE 1. Influence of different doses of TDZ or BA on adventitious bud initiation from *Stevia rebaudiana* leaf discs

Cytokinins (mg/L)		Shoot induction %	Number of shoots	Shoot length (cm)	Callus formation
TDZ	BA				
-	-	-	-	-	No
0.5	-	60	2.20±0.09^{bc}	3.32±0.07^{a}	No
1.0	-	70	1.10±0.05^{e}	3.10±0.10^{ab}	Small callus
2.0	-	65	2.00±0.04^{c}	2.92±0.13^{b}	Moderate callus
-	0.5	80	1.60±0.10^{d}	1.37±0.06^{e}	No
-	1.0	90	2.27±0.04^{b}	2.50±0.07^{c}	No
-	2.0	85	3.02±0.08^{a}	1.67±0.11^{d}	No

According to the Dunchans multiple range test (DMRT) at 0.5 level, the means ± SE (standard error) in each column followed by the same letters are not substantially different.

Shoot bud proliferation and PGRs combination

Different responses were observed (Table, 2) using different concentrations (0.5 and 1.0mg/L) of BA and TDZ in combination with different doses (0.1, or 0.5mg/L) of NAA or IAA for multiple shoot proliferation. After four weeks of culture, the optimum initiation of healthy shoot buds (10.40) with 3.15cm shoot length were observed using 1.0mg/L BA + 0.5 TDZ + 0.1 IAA combination (Fig. 1.A) followed by 1.0mg/L BA + 0.5mg/L TDZ + 0.5mg/L IAA combination that gave (8.37) shoots per explant and 2.20cm shoot length. Whereas, the tallest shoots were occurred using the combination of 1.0mg/L TDZ + 0.5 BA + 0.1 IAA recording 4.22cm shoot length (Fig. 1.B) followed by 0.5mg/L TDZ + 0.5 BA + 0.1 IAA combination that gave (3.85 cm shoot length). These results are in harmony with that of Deepu & Prasad (2007), Abd Alhady (2011), Deshmukh & Ade (2012). In contrast, The findings are in disagreement with Thiyagarajan & Venkatachalam (2012), who found that 1.0mg/L BA + 0.5 IBA combination was given a good growth of shoot bud 4.25 shoots with 5.02cm shoot length. Atalay et al. (2011), Aman et al. (2013), Singh et al. (2017) found that the addition of IBA along with BA was efficient using *Stevia rebaudiana* nodal explants, while IAA was proved the most suitable and efficient auxin for shoot bud multiplication compared to NAA.

On the other hand, increasing the concentration of NAA from 0.1 to 0.5mg/L in different combinations with 0.5 or 1.0mg/L of BA or TDZ, reduced *Stevia rebaudiana* shoot proliferation. However, Maharana et al. (2012) revealed that high concentration of cytokinins stimulated the development of meristems and the optimal concentration promotes shoot proliferation, and the inclusion of low concentration of auxins along with cytokinins triggers the rate of shoot proliferation. This differential response may be attributed to the specific age and physiological condition of the donor plant. The influence of cytokinins and auxins on optimizing shoot regeneration has been indicated in several species, such as *Catalpa ovata* (Lisowska & Wysokinska, 2000), *Echinaceae purpurea* (Koroch et al., 2002), and *Kigelia pinnata* (Thomas & Puthur, 2004).

Adventitious root formation

Adventitious root formation is not only a critical factor for the successful production of elite clones but also a key step in the vegetative propagation of woody, horticultural and agricultural plant species (Davis & Haissig, 1994). IAA and NAA significantly increased the number of roots per shoot and root length compared to the control (Table 3). Half strength MS with 2.0mg/L IAA seemed to be the optimum-rooting medium that gave a maximum root formation (100 %) with the highest number of roots per shoot (8.17), and with the highest root length (4.82cm). Then, adding 1.0mg/L IAA to the MS was found to come in second place that inducing (4.60) roots with 3.65cm root length.

TABLE 2. Influence of different concentration and combination of PGRs on adventitious bud regeneration from Stevia rebaudiana leaf discs after four weeks of culture

PGRs (mg/L)				Number of shoots	Shoot length (cm)
TDZ	BA	IAA	NAA		
0.5	0.5	0.1	-	5.55±0.09^{d}	3.85±0.10^{b}
		-	0.1	4.02±0.10^{ef}	1.87±0.12^{f}
		0.5	-	3.52±0.08^{g}	2.62±0.08^{d}
		-	0.5	2.12±0.12^{h}	1.67±0.13^{f}
	1.0	0.1	-	10.40±0.08^{a}	3.15±0.06^{c}
		-	0.1	6.32±0.08^{c}	1.62±0.08^{f}
		0.5	-	8.37±0.13^{b}	2.20±0.04^{e}
		-	0.5	4.25±0.11^{e}	1.82±0.08^{f}
1.0	0.5	0.1	-	6.17±0.13^{c}	4.22±0.04^{a}
		-	0.1	5.27±0.11^{d}	1.22±0.04^{g}
	1.0	0.1	-	5.45±0.09^{d}	2.30±0.04^{e}
		-	0.1	3.90±0.12^{f}	1.85±0.06^{f}

According to the Dunchans multiple range test (DMRT) at 0.5 level, the means ± SE (standard error) in each column followed by the same letters are not substantially different.

TABLE 3. Influence of different doses of IAA and NAA on Stevia rebaudiana root formation

Auxins (mg/L)		Root formation%	Roots no./explant	Root length (cm)	Callus formation
IAA	NAA				
-	-	-	-	-	No
0.1	-	90	2.85±0.15^{e}	1.37±0.10^{f}	No
0.5	-	95	4.17±0.11^{c}	3.07±0.13^{c}	No
1.0	-	95	4.60±0.12^{b}	3.65±0.10^{b}	No
2.0	-	100	8.17±0.14^{a}	4.82±0.11^{a}	Small callus
-	0.1	80	3.72±0.13^{d}	2.57±0.13^{d}	No
-	0.5	85	3.37±0.13^{d}	2.30±0.10^{d}	No
-	1.0	90	2.95±0.10^{e}	1.82±0.11^{e}	Small callus
-	2.0	95	2.40±0.12^{f}	1.57±0.13^{ef}	Small callus

According to the Dunchans multiple range test (DMRT) at 0.5 level, the means ± SE (standard error) in each column followed by the same letters are not substantially different.

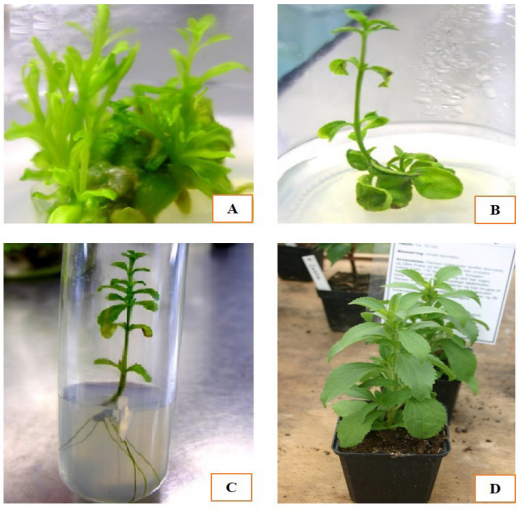

Fig. 1. Proliferation and acclimatization of multiple shoots from *Stevia rebaudiana* leaf explant [A: Shoot organogenesis on leaf explant of *Stevia rebaudiana*, B: Shoot elongation of *Stevia rebaudiana*, C: A rooted *Stevia rebaudiana* plantlet; D: *In vitro* propagated *Stevia plantlets* ready for field transfer

Similar findings are in line with Thiruvengadam & Jayabalana (2000), Jeyakumar & Jayabalan (2002), Deshmukh & Ade (2012), Anbazhagan et al. (2010) and Laribi et al. (2012) who reported that IAA was found to be more efficient and favorable for root formation.

However, increasing the concentration of IAA by more than 0.1mg/L was increased the root formation percentage, number of roots, and root length. Thus, the number of roots and its length were reduced with increasing NAA concentration to more than 0.1mg/L. This result was in harmony with Abd Alhady (2011). Small callus formation less than 5mm diameter was observed on MS medium supplemented with 2.0mg/L IAA and on MS with 1.0 or 2.0mg/L NAA. The results seems to be in harmony with those of Singh et al. (2017), Keshvari et al. (2018).

IAA in the concentration of 0.5mg/L in presence of three other concentrations (0.1, 0.5, or 1.0mg/L) of NAA, IBA, or 2,4-D has been investigated (Table 4). The optimum roots number (4.27) with 5.20cm root length was obtained on half-strength MS basal salts with 0.5mg/L IAA + 0.1mg/L IBA (Fig. 1.C). However, the formulation of 0.5mg/L IAA + 0.5mg/L IBA recorded the second-best root number (3.80) with (4.12cm) root length. The results showed that increasing IBA concentration or NAA from 0.1 to 1.0mg/L in combination with 0.5mg/L IAA decreased the roots number and its length. Moreover, the different 2,4-D concentrations presented the lowest values of root length and its number compared with the same concentrations of IBA or NAA. In addition, Increasing 2,4-D concentration of from 0.1 to 1.0mg/L in combination with 0.5 mg/l IAA increased the number of roots per shoot and root length. Finally, the in vitro plantlets grew very well in the greenhouse with survival rate of 60-80% (Fig. 1.D). These results seems to be in harmony with those of Attaya (2017), Keshvari et al. (2018).

Conclusion

An efficient and highly reproducible protocol has been made for the direct regeneration of several shoot buds from leaf explants to get healthy *Stevia rebaudiana* plants in a relatively short period and with a high survival rate for mass propagation and genetic improvement.

TABLE 4. Impact of different doses and combination of auxins on root formation of Stevia rebaudiana after four weeks of culture

Auxins (mg/L)				Roots no./explant	Root length (cm)
IAA	NAA	IBA	2,4-D		
	0.1	-	-	3.15±0.06^{d}	3.25±0.09^{d}
	0.5	-	-	2.57±0.04^{e}	2.92±0.06^{e}
	1.0	-	-	2.22±0.08^{f}	2.45±0.08^{f}
	-	0.1	-	4.27±0.07^{a}	5.20±0.09^{a}
0.5	-	0.5	-	3.80±0.07^{b}	4.12±0.06^{b}
	-	1.0	-	3.42±0.06^{c}	3.62±0.11^{c}
	-	-	0.1	1.30±0.07^{h}	1.40±0.09^{h}
	-	-	0.5	1.80±0.04^{g}	1.77±0.07^{g}
	-	-	1.0	2.32±0.08^{f}	2.47±0.08^{f}

According to the Dunchans multiple range test (DMRT) at 0.5 level, the means ± SE (standard error) in each column followed by the same letters are not substantially different.

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بروتوكول التكشف المباشر للبراعم الخضرية من زراعة الأوراق للاستيفيا لإنتاج شتلات حيوية

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تم اعداد بروتوكول تضاعف بسيط وفعال لإكثار نبات الاستيفيا باستخدام أوراق الاستيفيا أو أجزاء منها وبعض منظمات النمو النباتية المختلفة على بيئة موراشيجي وسكوج للحصول على نباتات صحية وذات معدل انتاج وفير. تم الحصول على معدل نمو خضري مثالي (90%) باستخدام 1 ملليجرام/لتر من البنزيل ادينين (BA) على بيئة موراشيجي وسكوج. كذلك فان أكبر عدد كبير من البراعم الخضرية (3.02) تكونت على المنفصل الورقي وبدون تكوين الكالس على نفس البيئة ولكن مضافاً اليها 2 ملليجرام/لتر من البنزيل ادينين بعد 4 أسابيع من الزراعة. كذلك تفوقت التوليفة المكونة من 0.5 ملليجرام/لتر من تي ديازورون (TDZ) مع 1 ملليجرام/لتر من البنزيل ادينين (BA) مضافاً اليها 0.5 ملليجرام/لتر من اندول حامض الخليك (IAA) والتي أعطت معدل 10.4 برعم خضري للمنفصل النباتي الواحد ومعدل طول للبرعم بلغ 3.15 سم.

كذلك فأن الأجزاء الخضرية الصحية ا لتي تمت استطالتها تم زراعتها على نصف قوة بيئة موراشيجي وسكوج المحتوية على الاملاح القاعدية وبدون الفيتامينات والتي ايضاً تحتوي على تركيزات مختلفة من اندول حامض الخليك ونفثالين حامض الخليك (NAA) منفردين أو في توليفة مع اندول حامض البيوتريك (IBA) او مركب 2،4 داي كلوروفينوكسي حامض الخليك (2,4-D) وكذلك معدل النمو الجذري المثالي ذات معدل جذور 8.17 لكل برعم خضري وبنسبة تجذير وصلت إلى 100% تم الحصول عليهم باستخدام نصف قوة بيئة موراشيجي وسكوج مضافاً اليها 2 ملليجرام/لتر اندول حامض الخليك منفرداً. وقد نمت الشتلات الصحية المتطورة والمتحصل عليها معملياً بنجاح لمدة 4 أسابيع واخذت تنمو طبيعياً في الصوبة الزجاجية.