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Direct organogenesis from cold treated *in vitro* leaf explants of *Stevia rebaudiana* Bertoni

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Abstract
Stevia rebaudiana is a perennial herb belonging to Asteraceae family and an excellent substitute of artificial sugar. Increasing demand of quality panting material has emphasized the need of rapid and mass multiplication through plant tissue culture technology in limited time duration. Sterilized *in vivo* leaves were used as leaf explants on different concentration of BAP, Kin and TDZ. In all media, shoot induction was not observed at any concentration from *in vivo* leaf explants. Cold treatment (4 ºC) of different time duration (0-72 hr) was applied on *in vitro* leaf explants and cultured on various concentration of TDZ (1.0-5.0 mg/l). Highest shoot induction response (42.40%) was occurred at 4.0 mg/l TDZ from 72 hrs cold treated *in vitro* leaf. Highest number of shoots per explant (25.80) was observed at 3.0 mg/l TDZ from 48 hrs cold treated *in vitro* leaf explants. Highest shoot length (1.36 cm) was observed at 3.0 mg/l TDZ from 72 hrs cold treated *in vitro* leaf. Cold treatment was used first time in Stevia which improve the direct organogenesis from *in vitro* leaf explants.

Keywords: cold treatment, leaf explants, TDZ, stevia

1. Introduction
Stevia rebaudiana Bertoni is worldwide accepted as an important medicinal plant belonging to Astereaceae family having valuable properties (Yadav *et al*., 2011; Singh *et al*., 2017a) [27, 28]. Leaves of Stevia produce natural sweetener which serve as an excellent substitute to artificial sugars for diabetic patients so it's known as "Calorie free Bio-sweetener of High Quality" (Preethi *et al*., 2011) [17]. The potential uses of Stevia which produces stevioside, a noncaloric sweetener that does not metabolize in the human body, hence control blood sugar level (Gantait *et al*., 2015) [3]. This is more important, especially in the context of the current social movement towards more natural foods.

In nature, seed germination in Stevia is poor and unsuccessful due to small endosperm and infertile seed (Carneiro *et al*., 1997; Goettemoeller and Ching, 1999) [2, 4]. Even some plant selections produce virtually infertile seed due to their self-incompatibility (Yadav *et al*., 2011; Singh *et al*., 2017) [27, 18]. Numbers of reports are available on successful conventional propagation method of Stevia via stem cuttings. But direct planting of stem cuttings in the field has limited success due to imperfect rooting (Yadav *et al*., 2011) [27]. Moreover, a huge number of stem cuttings are required for mass multiplication of plants which is an obstacle as number of mother plant need to be vanishing. Hence, conventional methods are not adequate for commercial production of Stevia plants and fulfill the current demand.

Micropropagation protocols of Stevia have been reported from different explants like leaf (Sivaram and Mukundan, 2003; Sreedhar *et al*., 2008; Jain *et al*., 2009) [24, 25, 11], nodal (Laribi *et al*., 2012; Modi *et al*., 2012; Thiyagarajan and Venkatachalam, 2012; Hassanen and Khalil, 2013; Lata *et al*., 2013; Singh and Dwivedi, 2013; Singh *et al*., 2014; Singh *et al*., 2017b) [12, 15, 26, 6, 13, 22, 21, 19] and shoot tips explants (Ibrahim *et al*., 2008a; Ibrahim *et al*., 2008b; Hassanen and Khalil, 2013) [9, 10, 6]. However, a major problem associated with *in vitro* culture is the possible occurrence of somaclonal variation through indirect organogenesis (Modi *et al*., 2012; Singh *et al*., 2014) [15, 21]. Literature survey revealed the occurrence of variability in tissue culture raised plants which did not produced true-to-type plants from leaf explants. Moreover, urgent need to the study about *in vivo* and *in vitro* leaf explants of stevia for direct organogenesis and assessment of genetic purity of regenerated plants by molecular markers for commercial exploitation require prime consideration.

Therefore, the present investigation was emphasized on development of an efficient reproducible *in vitro* micropropagation protocol for high frequency shoot multiplication of true-to-type plants from leaf explants and assessed the genetic uniformity of tissue culture raised plants of Stevia by molecular markers.

2. Materials and Methods

2.1 Source and preparation of explants

In the present investigation, rooted seedlings were collected from Jaipur, Jodhpur and Udaipur. These seedlings were grown under poly-house condition at High-tech horticulture farm, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and technology, Udaipur. Selection of the superior stevia plant on the basis of morphological and biochemical characteristics for tissue culture studies (Singh *et al*., 2017) [20]. A protocol for mass multiplication was established from nodal explants of *S. rebaudiana* (Singh *et al*., 2017b) [21].

Leaf explants (0.5-1.0 cm^2) were collected from 6 month old plants growing at Hi Tech Horticulture Farm, RCA, MPUAT, Udaipur. *In vivo* leaf explants were thoroughly washed in sterilized distilled water containing 1-2 drops of Tween-20 and then rinsed three times with sterilized distilled water. The explants were disinfected by treating with 0.4% Bavistin (Carbendazim 50% WP, BASF, India) for 15 min. with constant swirling at 110 rpm on shaker. Thereafter, the solution was drained and explants were rinsed properly 3-4 times with sterilized distilled water than explants were disinfected by treating with freshly prepared 0.1% mercuric chloride ($HgCl_2$) solution for 3 min for leaf explants with constant swirling at 110 rpm on shaker and then rinsed 3-4 times with distilled sterilized water. Surface sterilized *in vivo* leaves were cut into approximately 0.5-1.0 cm^2 size and inoculated in different concentration of media. For *in vitro* explants of leaf, prior established culture bottles (Singh *et al*., 2017b) [19] were kept in fridge at 4 ºC for different time duration (0-72 hr) and *in vitro* regenerated plants used to isolate leaf segments (0.5- 1.0 cm^2).

2.2 Nutrient medium and laboratory condition

Leaf explants were cultured on MS basal (Murasnige and Skoog, 1962) [16] medium fortified with different concentration of TDZ for shoot induction and BAP + Kin for shoot multiplication. Shoots were transferred on MS basal medium nourished with various concentrations of IBA, IAA and NAA for root induction. All media were supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar (Himedia, Mumbai, India). The pH of MS medium was adjusted to 5.80 prior to sterilization at 121 ºC for 15 minutes at 15 psi. The culture was incubated at 25±1 ºC with 16 h light/8 h dark photoperiod under the cool white fluorescent tubes (Crompton Greaves Limited, Mumbai). Subculture was done every three weeks interval with respective media. Rooted plantlets were placed in plastic pots, containing vermiculite and cocopeat (1:1) kept in plastic box to maintain high humidity. For the shoot multiplication, root induction, acclimatization and assessment of clonal fidelity employed following published procedure (Singh *el al*., 2017b) [19].

2.3 Statistical analysis

For *in vitro* tissue culture experiments, statistical analysis were performed with JMP software version 9 using Tukey Kramer HSD test to determines significant differences among treatment at $P \leq 0.05$.

3. Results and discussion

3.1 Direct shoot organogenesis from leaf explants

In this study, sterilized *in vivo* leaves were used as leaf explants on different concentration of BAP, Kin and TDZ. In all media, shoot induction was not observed at any concentration from *in vivo* leaf explants. Therefore, cold treatment (4 ºC) of different time duration (0-72 hrs) was applied on *in vitro* leaf explants. Cold treated *in vitro* leaf explants (0.5-1.0 cm^2) were placed on various concentration of TDZ (1.0-5.0 mg/l). Among all media, number of shoots from *in vitro* leaf explants with different concentrations was found significantly different after 5th week of culture. Highest shoot induction response (42.40%) was occurred at 4.0 mg/l TDZ from 72 hrs cold treated *in vitro* leaf. Highest number of shoots per explant (25.80) was observed at 3.0 mg/l TDZ from 48 hrs cold treated *in vitro* leaf explants (Table 1). Minimum number of shoots per explant (6.20) was recorded at 3.0 mg/l TDZ from zero hour cold treated *in vitro* leaf explant. Highest shoot length (1.36 cm) was observed at 3.0 mg/l TDZ from 72 hrs cold treated *in vitro* leaf and lowest shoot length (0.18 cm) was recorded at 4.0 mg/l TDZ from 12 hrs cold treated *in vitro* leaf.

In this study, cold treatment was used first time in Stevia which improve the direct organogenesis from *in vitro* leaf explants (Figure 1). Sivaram and Mukundan (2003) [24] reported that BAP found more effective than Kin for shoot induction from shoot apex, nodal and leaf explants. Whereas Kin showed shoot regeneration only in shoot apex and nodal explants. The maximum number of shoots, however, was observed in all the three explants on MS medium containing BA (8.87 mM) and IAA (5.71 mM). They further reported that maximum 11 shoots were regenerated from shoot apex followed by nodal (10) and leaf explant (8). The combination of BAP and NAA induced 3-4 shoots from shoot apex and nodal explants, but failed to initiate shoots in leaf explants. Kin in combination with either of the auxins, viz. NAA and IAA, completely failed to induce shoot formation in leaf explants while giving only one or two shoots in the shoot apex and nodal explants. Callus free, vigorously growing shoots were obtained in the case of shoot apex and nodal explants, whereas leaf explants could regenerate shoots after an intermediate callus phase. Shreedhar *et al*., (2008) [25] used MS basal media fortified with 8.88 µM BAP with Kin (4.56-6.98 µM) to achieve direct buds from both sides of midrib from adaxial surface of immature leaf explant of Stevia. Immature leaves of 0.6 to 1.0 cm were found to produce best response (93%) with a highest number of 4.93 shoot buds per explant. Pretreatment of low temperature (4 ºC) improved regeneration from *in vitro* leaf explants in present study. Significantly more regenerative shoots were obtained on leaf explants pretreated at 4 ºC for 48 hrs with average of 25.80 shoots per *in vitro* leaf explant at 3.0 mg/l TDZ. There was significant decrease in the number of shoots formed as a result of increasing the duration of low temperature (4 ºC) pretreatment of leaf explant. Improvement of shoot regeneration on other plant species was also reported in low temperature pretreatment (Hou *et al*., 1997; Singh *et al*., 2002; Andersone and Levinah, 2005; Guo *et al*., 2007) [7, 23, 1, 5]. The enhancement of plant regeneration by low temperature treatment was related to the alteration of endogenous auxin and cytokinin balace and redox-state which played a key role in plant growth and development (Huberman *et al*., 1997; Merce *et al*., 2003) [6, 14]. For the mass multiplication, root induction, field survival of regenerated plants and assessment of clonal fidelity employed following published procedure (Singh *el al*., 2017b) [19].

Table 1: Effect of cold treatments on adventitious shoot regeneration in *in vitro* leaf explants of *Stevia rebaudiana* was grown on MS basal media supplemented with different concentration of TDZ. Data was observed after 5-weeks.

TDZ (mg/l	Cold Treatment														
	0 hrs			12 hrs			24 hrs			48 hrs			72 hrs		
	% Shoots Induction	No of Shoots/ explant	Shoot Length (cm)	% Shoots induction	No of Shoots/ explant	Shoot Length (cm)	% Shoot induction	No of Shoots/ explant	Shoot Length (cm)	% Shoot induction	No of Shoots/ explant	Shoot Length (cm)	% Shoot induction	No of Shoots/ explant	Shoot Length (cm)
1.00	17.60 ± 2.03^{b}	3.60 ± 0.67^{ab}	0.62 ± 0.16^{a}	0.00 ± 0.00^{d}	0.00 ± 0.00^{b}	0.00 ± 0.00^{c}	6.40 ± 1.60^{c}	3.20 ± 0.80^{a}	0.38 ± 0.05^{a}	31.20 ± 0.80^{a}	2.60 ± 0.40^{c}	0.88 ± 0.10^{a}	32.80 ± 1.40^{ab}	6.40 ± 0.92^{a}	1.24 ± 0.15^{ab}
2.00	24.80 ± 1.95^{ab}	2.00 ± 0.44^{b}	0.72 ± 0.19^{a}	25.60 ± 1.60^{a}	10.20 ± 2.24^{a}	0.84 ± 0.11^{a}	13.60 ± 2.19^{ab}	0.80 ± 0.48^{a}	0.34 ± 0.20^{a}	19.20 ± 1.95^{cd}	8.40 ± 1.02^{b}	0.60 ± 0.10^{ab}	24.80 ± 1.95^{b}	4.80 ± 0.86^{ab}	0.64 ± 0.06^{ab}
3.00	28.00 ± 1.78^{a}	6.20 ± 1.52^{a}	0.56 ± 0.05^{a}	23.20 ± 1.40^{a}	3.40 ± 0.74^{b}	0.52 ± 0.07^{ab}	17.60 ± 4.56^{ab}	3.60 ± 1.36^{a}	0.58 ± 0.20^{a}	21.60 ± 2.71^{bc}	25.80 ± 1.52^{a}	0.50 ± 0.08^{ab}	37.60 ± 2.99^{a}	3.80 ± 0.58^{ab}	1.36 ± 0.16^{a}
4.00	20.80 ± 1.95^{ab}	1.40 ± 0.24^{b}	0.46 ± 0.09^{a}	9.60 ± 0.97^{c}	1.00 ± 0.63^{b}	0.18 ± 0.11^{bc}	11.20 ± 1.49^{bc}	1.40 ± 0.87^{a}	0.36 ± 0.24^{a}	10.40 ± 2.40^{d}	6.20 ± 0.86^{bc}	0.30 ± 0.08^{b}	42.40 ± 3.70^{a}	4.80 ± 0.48^{ab}	1.24 ± 0.21^{ab}
5.00	17.60 ± 1.60^{b}	2.80 ± 0.86^{ab}	0.50 ± 0.18^{a}	15.20 ± 1.40^{b}	1.60 ± 0.50^{b}	0.70 ± 0.19^{a}	20.00 ± 1.78^{a}	3.40 ± 0.74^{a}	0.48 ± 0.09^{a}	29.60 ± 2.03^{ab}	8.00 ± 1.22^{b}	0.68 ± 0.14^{ab}	8.80 ± 1.49^{c}	2.80 ± 0.37^{b}	0.70 ± 0.14^{ab}

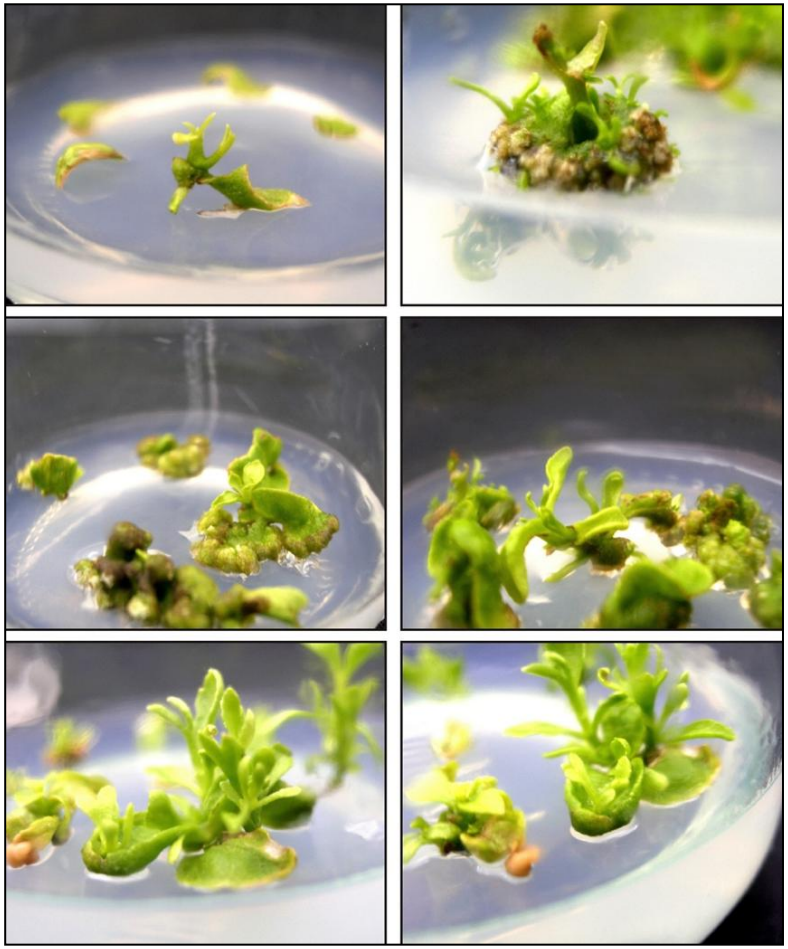

Fig 1: Shoot induction from cold treated *in vitro* leaf explants

4. Conclusion

In this study, an efficient reproducible protocol for *in vitro* mass multiplication from cold treated *in vitro* leaf explants of *S. rebaudiana* was established. Therefore, mass multiplication of planting materials can be increased by many folds using cold treated *in vitro* leaf explants and producing true-to-type quality planting material in limited time with high survival rate. Hence, the protocol can be considered for high frequency mass multiplication of quality planting materials of stevia for commercial exploitation.

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