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Establishment of an Effective *in vitro* Regeneration System for Sugar Beet (*Beta vulgaris* L.)

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Abstract

Plant regeneration and dedifferentiation are known to be difficult for sugar beet (*Beta vulgaris* L.) due to its recalcitrant nature. This leads to low transformation efficiency and thus successful application of plant molecular techniques is limited in sugar beet for its genetic enhancement. A prolific regeneration method has been established by modulating several plant growth regulators on the *in vitro* regeneration of *Beta vulgaris* var. V_6 KWS Serenada. Several types of explants excised from young seedlings of this variety were used for both direct and indirect regeneration of shoots. The highest response towards direct shoot formation and callus induction were obtained from cotyledonary nodes and hypocotyls, respectively. Explants were cultured on MS medium supplemented with different concentrations of BAP, GA_3 and 2,4 -D for callus induction as well as formation of shoots. Hypocotyls responded well for callus induction on MS medium containing 0.1 mg/l BAP + 2.0 mg/l 2,4-D, while cotyledonary nodes exhibited the highest responses towards shoot formation on MS medium containing 1.0 mg/l BAP and 1.5 mg/l GA_3. MS medium containing 2.0 mg/l IBA produced the highest number of roots per shoot. The *in vitro* raised rooted plantlets were successfully transferred to soil for acclimatization.

Introduction

Sugar beet (*Beta vulgaris*) is an herbaceous dicotyledon, belonging to the family Amaranthaceae (formerly Chenopodiaceae) and one of the four types of *Beta vulgaris* that are cultivated (Letschert 1994 and Ford-Lloyd 2005). It is the most significant industrial crop and one of the world's primary suppliers of sugar (Gurel et al. 2008, Maitah et al. 2016). The world produces around 145 × 106 t of sucrose annually, of which beet sugar

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and cane sugar are contributing almost 25 and 75 percent, respectively (Joersbo 2007). Sugar beet also has a considerable biomass production capability, it has been estimated that sugar beet can produce 5.7 m^3 ha^{-1} of bioethanol, compared to 2.6 m^3 ha^{-1} from wheat (Bekheet et al. 2008). Moreover, it also has significant economic contribution internationally such as cellulose-rich by-products from sugar beet pulp (Fishman et al. 2011) can be combined with various activators to generate material-activated carbon (Li et al. 2016, Samadi et al. 2009, Demiral et al. 2010).

The nutritional value of sugar beet is excellent, it contains 42.68 grams of calories, 8 grams of carbohydrates, 2 grams of fiber, and 1 gram of protein per 100 grams. Root is the main storage organ of sugar beet and when a beet root is harvested, it has been found that it contains around 75-76% water, 15-20% sugars, 2.6% non-sugars, and 4-6% pulp (Shrivastava et al. 2013). The two primary betalain pigments, red betanin and yellow vulgaxanthin I, are found in sugar beet, which has been thought to be the only source of betalains (Sadowska-Bartosz and Bartosz 2021). Although sugar beet is a biennial plant, modern cultivars are extremely heterozygous and naturally cross-pollinated, thus making it challenging to create new types by traditional breeding (Atanassov 1986, Bekheet et al. 2008). In Bangladesh, there are a few practical difficulties with sugar beet cultivation. In addition to the fact that free-living circumstances prevent flowering and seed production (Rahman et al. 2015). Moreover, sugar beets are prone to nematode infestation which leads to severe yield damage (Ghaemi et al. 2020).

Therefore, there is ample chance of applying modern biotechnology that will help the breeder to introduce salient features into sugar beet genotypes that are commercially useful. Development of a sugar beet micropropagation system and an *in vitro* regeneration technique are seen to be essential stages in the genetic modification of the plant using contemporary biotechnology. Several attempts have been made in this regard to advance sugar beet regeneration. Numerous sources of *B. vulgaris* organogenesis have been documented, including callus (Tetu et al. 1987 and Ritchie et al. 1989), leaf tissue (Ferytag et al. 1988), suspension culture (Van Geyt and Jacobs 1985), and protoplast (Bhat et al. 1986). Regardless, many investigations revealed that sugar beet plant regeneration is limited and strongly controlled by genotype and depends on the concentrations of phytohormones used in the nutrient medium (Krens and Jamar 1989, Subrahmanyeswari et al. 2022).

Although *in vitro* culture of many plants has made substantial progress recently, sugar beet tissue culture is not particularly prolific (Gurel and Gurel 2013). Because of its very heterozygous character due to outcrossing, a high degree of genotypic variability presents a considerable barrier to improvements in both regeneration and survival perspectives (Subrahmanyeswari et al. 2022). Moreover, traditional plant breeding techniques are labor- and time-intensive in producing new sugar beet genotypes/varieties. To produce sugar beet cultivars resistant to diseases, pests, and herbicides, traditional sugar beet breeding methods have been combined with cutting-edge *in vitro* culture and genetic transformation technology (Gurel et al. 2008, Rahman et

al. 2015). The quality, stress tolerance, and production of sugar beet might all be greatly increased with the availability of appropriate genetic transformation technology. Therefore, the main goal of this study is to create an effective *in vitro* regeneration procedure for the sugar beet variety V_6 KWS Serenada, which will further be utilized to create a plant genetic transformation protocol with the desired properties.

Materials and Methods

The investigation was carried out at the Plant Breeding and Biotechnology Laboratory, Department of Botany, University of Dhaka, Bangladesh. The seeds of *Beta vulgaris* var. V_6 KWS Serenada obtained from Bangladesh Sugar crop Research Institute (BSRI), Ishurdi, Pabna, Bangladesh, were used in this investigation.

For sterilization, seeds were washed with sterilized distilled water then immersed in 70% ethanol for 1 minute, followed by washing with autoclaved distilled water again. After that, these seeds were surface sterilized for 20 minutes using 30% commercial Clorox (containing 5.25% sodium hypochlorite). Then these seeds were again washed with sterilized water 5 times. After that, seeds were kept in distilled water for 24 hours. After 24 hours, seeds were transferred to germination medium containing full strength MS, P^H adjusted with 5.8, using 0.8% agar and 3% sucrose. Explants were excised from 13-15 days old seedlings of *Beta vulgaris* var. V_6 KWS Serenada and were inoculated on MS media supplemented with varying quantities and combinations of BAP, GA_3 and 2,4-D for shoot induction.

In vitro regenerated shoots were moved to half-strength MS with varying IBA and IAA concentrations (1-2 mg/l). In a growth chamber at 24 ± 1ºC, all cultures were inoculated under a cool white, fluorescent light with 16-hour light/ 8-hour dark photoperiod. Once the roots had grown sufficiently for acclimatization, the plantlets were transferred to little plastic pots with autoclaved soil.

Results and Discussion

Plant biotechnology has the potential to significantly improve quality and stress tolerance through successful gene transfer methods. It could be an affordable and sustainable way to manage pests and diseases (Nyaboga et al. 2015). Moreover, by using genetic engineering to develop new varieties of sugar beet (*Beta vulgaris* L.) which are more suited to harsh environmental conditions and diseases (Moazami-Goodarzi et al. 2020). Therefore, it is important to establish an effective regeneration system, particularly for a crop like sugar beet (*Beta vulgaris* L.) which is highly recalcitrant.

The germination of sugar beet was quite time consuming due to its hard seed coat. The outer coat of beet seeds was too hard to split open and extract the entire seed. Furthermore, beet seeds have a lot of cracks on their surface that trap germs and contaminants and lessen their disinfecting power (Zhang et al. 2023). To overcome such

challenges, in this investigation, the seeds were sterilized with 30% commercial Clorox and kept in sterile water for 24 hours and after that, seed coats were removed by using sterile forceps and scalpel. All these methods had a positive effect and significantly reduced the pollution rate (Rahman et al. 2015).

For germination, sterilized seeds of V_6 KWS Serenada were placed on full strength MS medium supplemented with vitamins including myoinositol, nicotinic acid, thiamine, and pyridoxine. From the germination data, it was observed that the rate of germination was quite higher in V_6 KWS Serenada and it was approximately 75-80%.

In sugar beet regeneration, explants play a crucial role (Krens et al. 1989). In this study, explants were collected from 13-15 days old seedlings (Fig. 1b). To identify the ideal explants, five distinct types of explants including leaf with petiole, hypocotyls, cotyledonary leaf, petioles, and cotyledonary nodes were used. All these types of explants were incorporated into three different types of shoot induction media containing MS supplemented with 0.1 mg/l BAP + 2.0 mg/l 2,4 D; 1.0 mg/l BAP + 1.5 mg/l GA_3 and 2.0 mg/l BAP + 1.0 mg/l GA_3. Obtained results suggested that the percentage of direct organogenesis from cotyledonary node explants was much greater than other explants (Table 1). The capability of generating new shoots without an intermediary callus phase made cotyledonary node explants a better choice for sugar beet transformation (Lindsey and Gallois 1990). It is easier to avoid somaclonal variation by direct regeneration in compared to callus-based transformation techniques from cotyledonary node explants (Ergül et al. 2018).

Table 1. Responses of different types of explants of *Beta vulgaris* var. V_6 KWS Serenada in case of shoot formation.

Types of explant	No. of explants inoculated	No. of responsive explants	% of regenerative explants
Leaf with petiole	80	40	50
Hypocotyls	80	No response	No response
Cotyledonary leaf	80	52	65
Petioles	80	32	40
Cotyledonary node	80	68	85

Among the explants, only hypocotyls responded well in callus induction, indicating that 2,4-D was more appropriate for indirect organogenesis, and it takes about 25-30 days for callus initiation. The addition of 0.1 mg/l BAP + 2.0 mg/l 2,4-D medium produced the highest callus induction percentage in Toucan (*Beta vulgaris*) genotype (Morsi et al. 2019). However, this callus did not produce any shoots.

Therefore, further studies were carried out with cotyledonary node explant. The frequency of shoot formation was observed about 80-90% in MS media supplemented

with 1.0 mg/l BAP + 1.5 mg/l GA_3 and the media containing 2.0 mg/l BAP + 1.0 mg/l GA_3 showed 44-67% shoot formation after 15 days of responses (Table 2). Various stages of formation of shoots have been presented in Fig. 1. Similar findings were observed by Mishutkina and Gaponenko (2006), Ergül et al. (2018) and Gurel et al. (2019) who found that 0.5-1 mg/l, 1-3 mg/l and 1-2 mg/l BAP, respectively had a considerably positive impact on the rates of regeneration of both wild and cultivated sugar beet plants. Several studies have revealed that varied amounts of BAP are good for regeneration rates. This could be because various sugar beet genotypes were employed in each study (Gurel et al. 2019).

Derived shoots were then cultured on half strength of MS supplemented with four different combinations of auxins including 1.0 mg/l IAA, 2.0 mg/l IAA, 1mg/l IBA and 2.0 mg/l IBA. Among these combinations, the highest number of root initiation (75%) was observed on the media containing 2.0 mg/l IBA. In contrast, no responses were exhibited on the media containing 1.0 mg/l IAA (Table 2). In sugar beet, among different treatment combinations, the half strength MS media showed the highest percentage of rooted plants (31.67%) on 2.0 mg/l IBA, while the treatment with 4.0 mg/l IBA showed a considerably lower percentage of rooted plants (Rahman et al. 2015).

Table 2. Effects of different growth regulators on initiation of shoots and roots in *Beta vulgaris* var. V_6 KWS Serenada.

Medium	Growth regulators (mg/l)		Shoot formation	
	BAP	GA_3	% of responsive explants towards shoot formation	Mean no. of shoots/explant
Shoot-induction medium-I	2.0	1.0	44-67%	2.07 ± 0.605
Shoot-induction medium-II	1.0	1.5	80-90%	3.33 ± 0.67
			Root formation	
	IAA	IBA	% of responsive explants towards root formation	Mean no. of roots/shoot
Root-induction medium-I	2.0	-	41%	2.3 ± 0.67
Root-induction medium-II	-	2.0	75%	5.08 ± 0.65
Root-induction medium-III	1.0	-	No response	0
Root-induction medium-IV	-	1.0	60%	3.0 ± 0.65

It was also observed that only 8-10 days were required for root initiation. According to Chhun et al. (2003), IBA was more resilient in solutions and has a far greater impact on starting rooting than IAA. Once their roots had fully developed, plantlets were successfully transplanted into tiny plastic pots filled with autoclaved soil (Fig. 1i).

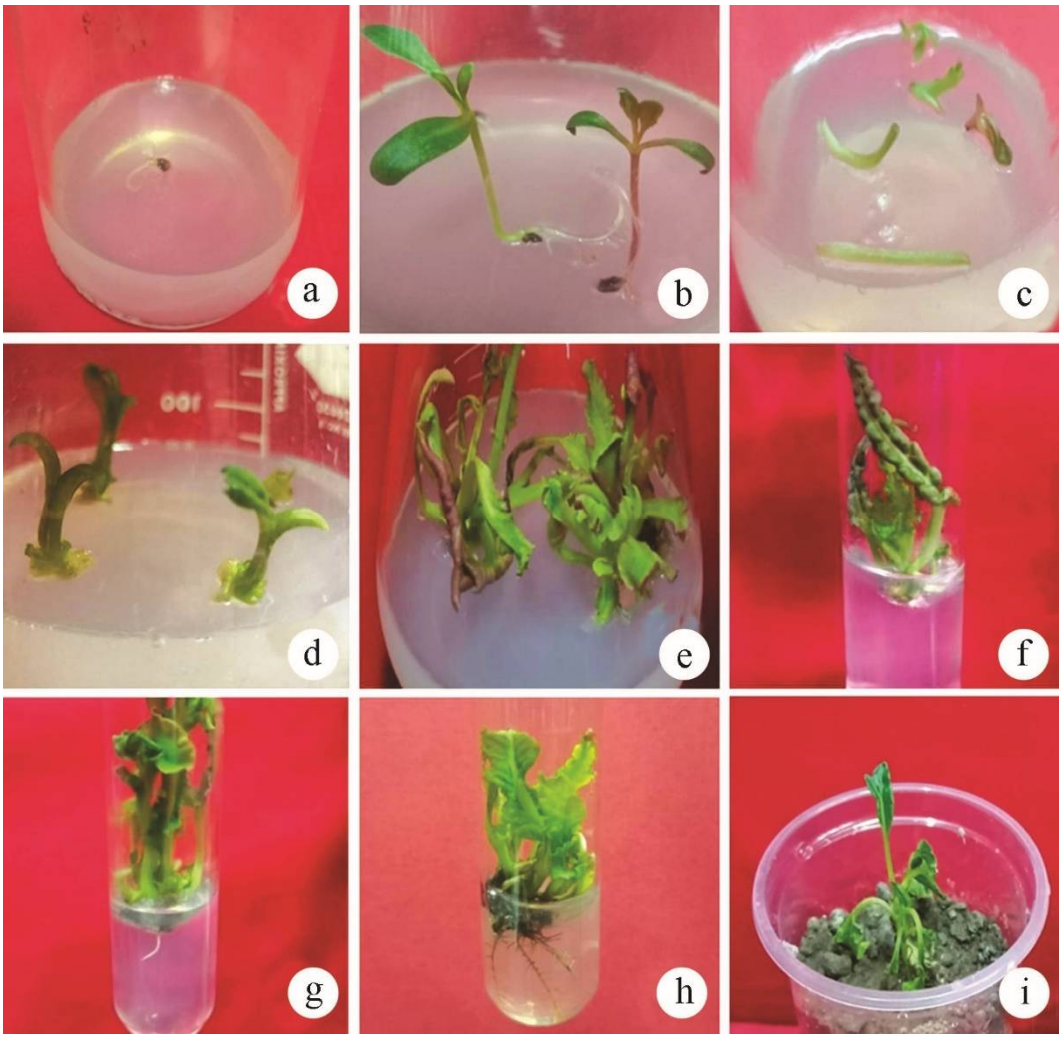

Fig. 1(a-i): Different stages of *in vitro* regeneration of Sugar beet from cotyledonary node of V_6 KWS Serenada. (a) Germinating seed, (b) 15 days old seedlings, (c) Cotyledonary node explants, (d) Initiation of shoot after 7 days on shoot induction medium, (e) Elongation of multiple shoots on shoot elongation medium, (f) Shoot on root induction medium, (g) Initiation of root after 9 days on root induction medium, (h) Formation of roots on rooting medium, (i) Acclimatized plants observed 20 days after their transfer from *in vitro* rooting medium to soil.

Sugar beet (*Beta vulgaris*) is an important sucrose-producing crop worldwide. Although there has been significant advancement in the past ten years regarding the introduction of foreign genes into crops, sugar beet continues to be recalcitrant to genetic modification (Kishchenko et al. 2004). Through this study, an efficient *in vitro* regeneration process for *Beta vulgaris* var. V_6 KWS Serenada was produced, which can be further utilized for genetic transformation in incorporating agronomically significant genes in sugar beet.

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