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Original Research Article

In vitro regeneration of Sugar Beet (*Beta vulgaris* L.) via leaf explants and callusing

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Abstract: Sugar beets (*Beta vulgaris* L.) represent a major segment of the sweetener industry in the United States. The Southwest region and the Texas portion of the Great Plains region are the only ones that have experienced a decline in production in the past 15 years. Although numerous factors have contributed to this decline, in a large part it has been due to rhizomania, a devastating disease of sugar beets caused by *Beet Necrotic Yellow Vein Virus* (BNYVV). The objective of this research was to develop a standardized reproducible technique to regenerate plantlets via *in vitro* culture that can be used for screening the disease resistant varieties of this important crop. Various combinations and concentrations of auxins and cytokinins were tested to induce dedifferentiation of the somatic cells. We recorded the regeneration of plantlets directly from the leaf explants. The leaf explants showed shooting and rooting directly when cultured on IAA (0.5 mg/l) and BAP (2 mg/l). The callus culture with 6, BAP (1.5 mg/l) and IAA (0.2 mg/l) led to the formation of the heart shaped or "T" shaped or "Y" shaped embryoids that exhibited high level of totipotency. The embryoids followed rooting in MS medium supplemented with IAA (0.2 mg/l). Root formation and shoot formation either directly from the explants or from regenerable callus were observed. Modification and refinement of *in vitro* techniques allowed us to obtain regenerable callus that had the capability of entire plant regeneration. This technique was successfully implemented to regenerate plantlets that showed 63% survival.

Keywords: Plant Regeneration, Somatic Embryoids, Sugar Beets, Tissue Culture.

Introduction

Micropropagation is an important alternative to more conventional methods of plant propagation. It involves production of plants from very small plant parts (e.g. buds, nodes, leaf segments, root segments etc.), grown aseptically in a container where the environment and nutrition can be controlled (Zilkah *et al.,* 1999). Sugar beets (*Beta vulgaris* L.) represent a major segment of the sweetener industry in the United States. The National Agricultural Statistics Service estimates an annual sugar beet yield of 32.521 million tons in 1.378 million acres of harvested area. With a yield estimate of 23.6 tons per acre beets outpaced sugarcane by almost 50% of the 2001 nation-wide sugar production. In the United States, sugar beets are produced in five distinct geographic regions (Rush *et al.,* 1995).

1. The Great Lakes region, (Michigan and Ohio);
2. The Red River Valley region (North Dakota and Minnesota) which constitutes the most intensive sugar beet production area, producing 46.22% of the annual national crop;
3. The Great Plains (Montana, Wyoming, Nebraska, Colorado, New Mexico);
4. The Northwest region (Idaho and Oregon);
5. The Southwest Region (California)

Of these regions mentioned above, the Southwest region and the Texas portion of the Great Plains region are the only ones that have experienced a decline in production in the past 10 years (Sugar and Sweeteners Yearbook, 2000, 2001). Although numerous factors have contributed to this decline, in a large part it has been due to rhizomania, a devastating disease of sugar beets caused by *Beet Necrotic Yellow Vein Virus* (BNYVV) (Rush *et al.,* 1993, 1994, 1995).

Most sugar beet cultivars grown in the Imperial Valley of California possess the *Rz* gene that confers resistance to rhizomania, a disease caused by BNYVV. BNYVV is transmitted by the obligate parasite *Polymyxa betae*, which survives in field soils and in roots of infected sugar beets. Recently, fields planted to these resistant cultivars began to show typical symptoms of Rhizomania.

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Micropropagation has the ability to yield plant parenchymal cells, callus, and somatic embryos that can be studied and bioengineered, giving solutions to the sugar industry and growers that would eliminate the need for costly management regimens (Dovzhenko, 2003). Sustainability of sugar production from sugar beets is of significant economic importance. The National Agricultural Statistics Service (NASS) published 2005 crop year (FY 2006) sugar beet acreage intentions for planted area at the end of March, and acreage intentions were about 3.5 percent lower than 2004 crop year area planted. (Sugar and Sweeteners Outlook, 2005)

Despite significant breakthroughs in DNA technologies sugar beets are recalcitrant and are not routinely easy to reproduce. "Recalcitrant species" are plant species that are difficult either to regenerate using tissue culture methods or to transform with foreign DNA. The narrow gene pool of sugar beets has led to the reliance on a single gene for disease resistance. This has many implications genetically, to include the potential loss of a significantly important food crop.

Tissue culture has the ability to yield plant parenchymal cells, callus, and somatic embryos, which can be studied and bioengineered. These studies can give solutions to the sugar industry and sugar beet growers that would eliminate the need for costly management regimens, to include disease management and decreases in production. Maintaining the sustainability of significantly important food crops can be attained through the use of molecular technique and *in vitro* tissue culture of sugar beets. The objective of this *in vitro* culture was to maximize a regeneration regimen that would allow for future bioengineering of this economically important food crop.

Materials and Methods

Beta-1395 sugar beet seeds were surface sterilized and planted on (Murashige and Skoog, 1962) culture medium to initiate germination and seedling growth. The objective was to obtain explants that would be used to develop various types of calli. To induce callusing we used Murashige and Skoog's (MS) culture medium modified with various concentrations and combinations of growth supplements as follows:

1. 750 ml double distilled water
2. 30 g sucrose
3. 0.5% w/v PVP (Polyvinyl-pyrrolidone)
4. 2 mg glycine
5. 4.4g Murashige Minimal Organic Medium
6. 0.5 mg nicotinic Acid
7. 0.5 mg pyridoxine

Various concentrations and combinations of growth factors like, auxins and cytokinins and growth supplements like coconut milk were added to modify the MS medium to induce morphogenesis. MS seed medium was used to germinate the viable seeds. Callus production was anticipated on MS C media and MS D media (Table.1). Table-1 shows the variations that were made to the MS medium (Murashige and Skoog, 1962).

The culture medium was adjusted to a pH of 5.6~5.8, then autoclaved for 20 min at 120ºC. Liquid media were poured evenly into previously autoclaved culture tubes. The tubes were set in a rack for solidification. Tubes containing MS S medium were placed vertically in a test tube rack for solidification and all other medium was allowed to solidify on a 45^{o} slant for increased surface area.

Table.1: Modifications to MS Media

Supplements for Modification	MS Media MS	Callus "C" MS C	Callus "D" MS D	Rooting "R" MS R	Seed "S" MS S
3-IAA (auxin)		1 mg/l	5 mg/l	2 mg/l	0
6-BAP (cytokine)		4 mg/l	1 mg/l	0.5 mg/l	0
Agar (Tissue Culture Grade)	4.5 g/l	4.5 g/l	4.5 g/l	1.5 g/l	2.0 g/l
Coconut Milk		80 ml/l	80 ml/l	80 ml/l	0
Sucrose	3%	3%	3%	3%	3%

Legends used here: MS= Murashige and Skoog's medium, 1962; C & D=Media for callusing; R= Medium for rooting; S=Medium for seed germination.

All cultural manipulations were done under a laminar airflow cabinet by following aseptic techniques in a standard tissue culture laboratory to avoid any microbial contamination. After surface sterilization one seed was implanted into every culture tube containing medium. Culture tubes were capped after flaming and wrapped with Parafilm to prevent any contamination. The tubes were placed in a rack in the culture room. The counter was cleaned with 0-Phenylphenyl to disinfect the workspace. We observed high level of contamination at the beginning of this research. The high rate of contamination could be attributed to the porous surface of the sugar beet seeds. Following steps were taken to maintain a full asceptic environment to reduce contamination.

1. Cleaned workspace with Amphyl Disinfectant (Reckitt Benckiser Professional)
2. Washed hands with soap and water then sprayed hands with alcohol.
3. Used 90% ethanol to flame the instruments maintaining sterilization on seeds and culture medium.
4. Planted one seed per culture tube containing medium.
5. Culture tubes were capped and wrapped with Parafilm M® (VWR Scientific) to reduce the chance of contamination.

Set the tubes in a rack, in the dark at 25ºC to allow them to grow.

Results and Discussions

The objective of this research was to induce morphogenesis from dedifferentiated cellular structures. The regulation of auxin and cytokinin to develop callus, somatic embryos, and protoplasts is done via growth hormone and supplements to standard MS growth medium.

Growth Factors used in this research fall into two classes. Auxins and cytokinins are used to force dedifferentiation of somatic cells. Indole-3-Acetic Acid (3-IAA) and 2, 4-Dichlorophenoxyacetic acid (2, 4-D) are the most widely used auxins.

Germination efficiency of sugar beet seeds was recorded and appeared to be consistent with Gurel (1997, 2001). Survivability of the germinated seedlings varied throughout the course of this research.

We analyzed rooting efficiencies on MS media and MS R media. An F test was conducted to determine if there was a significant difference between the 2 medium. For this design $\alpha=0.05$ was chosen. Our *p* value for the T test was 0.04 therefore rooting media has a greater significant effect than MS media on root regeneration (Table.1).

Table.2: Effect of media on rooting efficiency

MS Media	Rooting Media	
N	24	26
Sum	10	23
average	0.417	0.885
variance	0.601	1.146

Two types of calli were usually recorded; white and friable callus, or green and compact callus, referred to as Type I and Type II, respectively (Figs. 3C, 3D). Type I callus mostly consisted of large and translucent cells (Fig. 4 B), while Type II callus contained small green cells (Fig. 3D). These results were consistent with previous work done by both Dovzenko (2003) and Gurel (1997). Type I callus was usually formed on medium containing 1 mg/l 6-BAP and 5 mg/l 3-IAA, or on medium containing no plant growth regulators (i.e., hormone-free medium). However, Type II callus often developed on media containing 4 mg/l 6-BAP and 1mg/l 3-IAA as seen by Dovzhenko (2003).

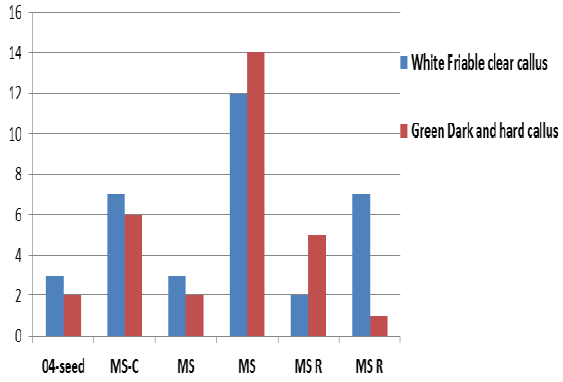


Fig.1: Rate of two types of callus production in *Beta vulgaris* in different media

Only friable (soft, nodular) type I callus of sugar beets was found to have regeneration activity (Fig.1) Regeneration activity of Type I callus was noted by Krens *et al.,* (1990). Non-regenerable Type II callus displayed different morphologies: white, or brown, or colorless soft callus which consisted of enlarged elongated cells and compact white, or brown, or colorless callus (Fig. 3D). Type II callus yielded no specific tissue regeneration ability in our research. The lack

of ability of Type II callus regenerating specific tissues or entire plants is supported by Gurel (1997, 2001) and Dovzhenko (2003).

Root formation and shoot formation either directly from the leaf explants or from regenerable callus were observed (Figs. 2A and 2C). Shoot organogenesis was seen more often than rooting from Type I callus as observed by Gurel (1997). We recorded a balance rooting among the explants that were transferred to the rooting media MS R that had a combination of specific hormones that induced profuse rooting.

Figure.2A: Seed germination of *Beta vulgaris* on ½ MS Medium and callusing and rooting from the leaf explants. **Fig.B:** Leaf explants shows callusing and anthocyanin accumulation. **Fig.C:** Rooting from a leaf explant indicating the possibility of direct regeneration from leaf explants. **Fig.D:** Multiplication of shootlets. **Fig.E:** isolated regenerated shootlets. **Fig.F:** Rooting from microshoots.

IAA (0.5 mg/l) and BAP (2mg/l) were used to modify the MS medium to induce de-differentiation of somatic cells. Rooting was obtained by using IAA and IBA (0.5 mg/l) Root and shoot formation occurred directly from the leaf explants or from regenerable callus were observed. Modification and refinement of *in vitro* techniques allowed us to obtain regenerable callus that had the capability of entire plant regeneration.

Using callus we established the cell suspension culture to obtain protoplast and further experimentation. The morphogenesis process was studied using light and fluorescent microscopy. Staining the cultured cells with vital stain Evan's Blue helped us to screen the regenerative cells from suspension culture. We observed the torpedo shaped embryonic initial that exhibited characteristic fluorescence with FITC filter. The slides prepared from the actively grown culture were observed under the high power and oil immersion lenses and photographs were taken using an Olympus BX40 microscope equipped with FITC, TRITC filters and an Olympus DP-70 digital camera connected to the computer with Image Pro 6.0 software.

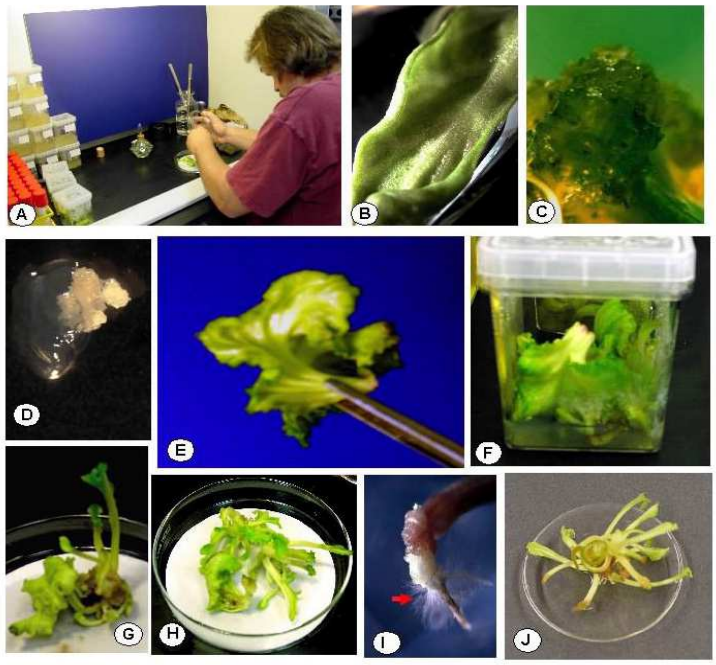
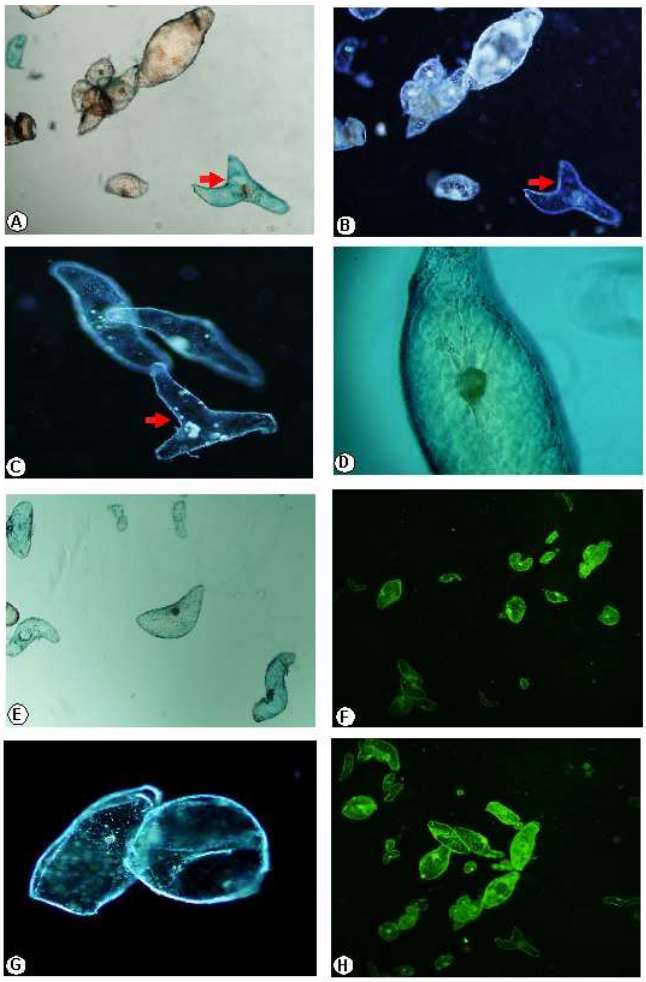


Figure.3A: Aseptic manipulation under laminar air flow **Fig.B:** Leaf explants collected from germinated seedling of *Beta vulgaris*. **Fig.C:** Growth of green compact Type II callus. **Fig.D:** Showing the White Type I callusing. **Fig.E:** Inoculation of grown leaf explant into the culture medium. **Fig.F:** Subculturing. **Fig.G:** Shoot multiplication for mass clonal propagation. **Fig.H:** Microshoot with induced rooting. **Fig.I:** Enlarged view of roots. **Fig.J:** A Complete plantlet developed from microshoots.

The heart shaped, "T" shaped or "Y" shaped cells exhibit the highest level of totipotency (Figs. 4A, B, and C). The totipotency of the heart shaped cells was first observed and reported by Rietsema (1953) in suspension cultures isolated from *Datura spp.* L., demonstrated a bipolar cell. We observed such cells in the culture with cytoplasmic fibrils on the callus tissue (Fig. 4D). Observation of the callus suspension culture showed a "Y" shaped cell, a bipolar cell, a

unipolar torpedo shaped cell, and a round meristematic cell (Figs. 4 E, G).

The bipolar nature of somatic embryos (Figs. 4A, B,C) can give rise to both shoot and root tissue (Figs. 2E and F) as was recorded by Singh (1998). The disaggregated mass of cells was formed because of breakage of cytoplasmic connections with other cells (Fig. 4H). These cells have cutinized their outer walls allowing the isolation of a specific morphology. Singh (1998) addressed this phenomenon as a vital one to plant biotechnology.



Fluorescent Microscopy on cultured cells from Beta vulgaris using Evans Blue and Fluorescin. Fig. 4 A.View of callus suspension culture (10x), arrow pointing the Torpedo shaped embryo. Fig.B and C.Torpedo shaped embryo under fluorescence. Stained with Evans Blue, observed under Phase Contrast (10x, 40x). Fig. D. A cell from suspension culture (40x) showing cytoplasmic fibrils (40x). D. Viable cells stained with Evans Blue. Figs. F. and H. Evans Blue with FITC filter. Fig.G Dividing meristematic cells stained with Evans Blue.

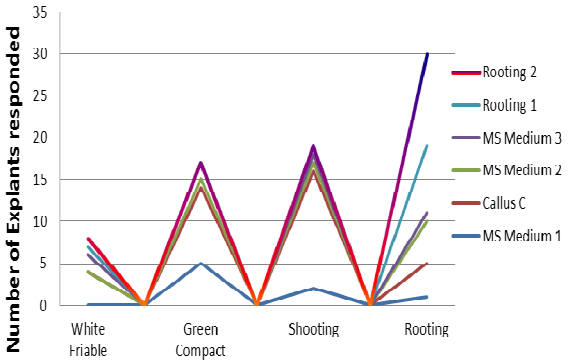


Fig.5: Morphogenesis and organogenesis in tissue media

Because the regeneration capacity of hypocotyl or cotyledon derived friable callus was reported earlier (Catlin, 1990; Jacq, 1992), we concentrated on the developing a reproducible and efficient method for regenerating an entire plant from cultures of regenerable callus inducted from seedling explants. Modification and refinement of *in vitro* techniques allowed us to obtain regenerable callus that had the capability of entire plant regeneration (Figs. 3A-J).

Age of the culture and photoperiod had an effect on Type I callus formation. Hypocotyl and cotyledon explants from seedlings of different age (three and five-weeks old) that were germinated either in the darkness or in a 16hr./day photoperiod were tested for their callus induction on MS C medium either in the darkness or in a 16hr./day photoperiod. The best callus formation efficiency was observed when hypocotyl explants from five-weeks-old etiolated seedlings were cultured in dark.

The method described here may be used for the consistent propagation of large numbers of plantlets from explants of sugar beet. The high regeneration shoot data obtained reflect the simulative effect of appropriate levels of 3-IAA and 6-BAP on shoot organogenesis as envisaged in the present investigation (Fig. 5) and by other authors previously (Skoog, 1948; Tetu, 1987). A number of workers have reported that 6-BAP induced shoot formation in sugar beet, including adventitious shoot formation from leaves following the spraying of 6-BAP to the cotyledons (Saunders, 1982). As evident in the Fig. 5 highest frequency of shooting was recorded in the regenerating callus (green compact) with Callus C medium and

profuse rooting was recorded with Rooting 2 medium.

Table.3: Rate of biomass production

	Sum of Squares	df	Mean Square	F	Sig.
Between Media Types	0.226	2	0.113	4.596	0.016
Within Groups	0.910	37	0.025		
Total	1.137	39			

We recorded a significant interaction between organogenesis and media type at a $p<0.05$. The effect of hormone analog concentrations was seen in Table 3. We looked at five categories of organogenesis: shooting, rooting, callus, and two different combinations of single organogenesis. As seen previously by Raven, (1992), Salisbury *et al.,* (1995) there is an influence of hormone and hormone analogs on specific organogenesis.

Conclusion

When subcultured repeatedly an exponential increase of the production of shootlets occurred on fresh media for a four - five months after which there was a sharp decline in regeneration efficiency. The number of shoot buds produced varied with the concentration and combination of growth factors used for subculturing (Fig. 5). After seven months of culture the regenerative potential declined by a significant level, similar to the findings of Franclet and Boulay, (1989). It was noticed that long term culture of another leguminous tree *Dalbergia sissoo* Roxb., resulted in an accumulation of genetic errors (Ghosh et al. 1992a, 1992b, 1998). Microshoots 4-5 cm long were transferred to two different sets of medium. One set was agarified half strength MS, the other was liquid half strength MS. Filter paper bridges were used in culture vessels with liquid half strength of MS, upon which shootlets were placed to achieve rooting with the Rooting 1 and 2 media (Fig. 5). In the next step the plantlets were transferred to sterile potting soil. The plants were about 20 - 22 cm long after 6 weeks when they were transferred to larger pots in the green house. Following the transfer from the culture room to pots, in average 63% plants survived.

References

1. Catlin DW, The effect of antibiotics on the inhibition of callus induction and plant regeneration from cotyledons of sugarbeet (*Beta vulgaris* L.). Plant Cell Rep 1990, 9,285-288.
2. Davies PJ, Plant Hormones: Physiology, Biochemistry and Molecular Biology. 1995, Dordrecht: Kluwer.
3. Lovzhenko A., Hans-Ulrich Koop. Sugar beet (*Beta vulgaris* L.): shoot regeneration from callus and callus protoplasts. Planta 2003, 217, 374–381.
4. Franclet A, Boulay M *In:* JS Pereira and JJ Landsberg (eds.). Biomass production by fast growing trees Kluwer Acad. Pub. Dordrecht. 1989, 267-274.
5. Ghosh N, Chatterjee A, Cytological studies on the effect of gamma radiation on *Albizia procera* Benth. *In:* G.K. Manna and S.C. Roy (eds.). Perspectives in Cytology and Genetics 1992, 7, 1199-1209.
6. Ghosh N, Chatterjee A, Cytological study on the effect of aging on callus cultures of *Dalbergia sissoo* Roxb. Plant Sciences in the Nineties, 1992, 440-447.
7. Ghosh N, Chatterjee A, Sinha A, Smith DW, Somaclonal variation in *in vitro* culture of *Dalbergia sissoo* Roxb. - a leguminous tree. 1998. GK Manna and SC Roy (eds.). Perspectives in Cytology and Genetics 1998, 9: 685-696.
8. Gurel E, Callus and root development from leaf explants of sugar beet (*Beta vulgaris* L.): Variability between cultivars, plants and organs. Turkish J Bot 1997, 21: 131-136.
9. Gurel S, Gurel E, Kaya Z, Callus development and indirect shoot regeneration from seedling explants of sugar beet (*Beta vulgaris* L.) cultured *in vitro*. Turkish J Bot 2001, 25: 25-33.
10. Jacq B, Tetu T, Sangwan RS, Laat AD, Sangwan-Norreel BS, Plant regeneration from sugarbeet (*Beta vulgaris* L.) hypocotyls cultured in vitro and flow cytometric nuclear DNA analysis of régénérants. Plant Cell Rep 1992, 11:329-333
11. Krens FA, Jamar D, Rounwendal GJA and Hall RD., Transfer of cytoplasm from new Beta CMS sources to sugar beet by asymmetric fusion. 1. Shoot regeneration from mesophyll protoplasts and characterization of regenerated plants. Theor Appl Genet 1990, 79: 390-396
12. Murashige T and Skoog F, A revised medium tissue cultures. Physiol Plant 1962, 15: 473-497

13. Raven PH, Evert, RF, and Eichhorn, SE, Biology of Plants. New York: Worth. 1992, 545-572.

14. Rietsema J, Satina S, and Blakeslee AF, The effect of sucrose on the growth of *Datura stramonium* embryos in vitro. Am J Bot, 1953, 40: 538–545.

15. Rush CM, French RC, Heidel GB, Texas 7 a possible strain of beet necrotic yellow vein virus. 1993, 59-62 in: Proc. Symp. Int. Work. Group Plant Viruses Fungal Vectors, 2nd Montreal Canada.

16. Rush CM, French RC, Heidel, GB, Differentiation of two closely related Furovirus using the polymerase chain reaction. Phythopatology 1994, 84, 1366-1369.

17. Rush CM, Heidel GB, Furovirus Diseases of Sugar Beets in the United States. Plant Dis. 1995, 79,868-875.

18. Saunders JW, Mahoney M, Benzyladenine induces foliar adventitious shoot formation on young plants of 2 sugar beet (*Beta vulgaris* L.) cultivars. Euphytica 1982, 31, 801–804.

19. Singh BD, Biotechnology. Kalyani Publishers. Calcutta, India. 1998, 204-47.

20. Skoog F, Tsui C, Chemical control of growth and bud formation in tobacco stem segments and callus cultured *in vitro*. Am J Bot 1948, 35, 782–787.

21. Salisbury FB, Ross CW, Plant Physiology, 1995, 3rd Edition, Wadsworth, Belmont, California, 197.

22. Sugar and sweeteners yearbook, production by commodities, Economical Research Service, U.S. Department of Agricultural Washington, D.C. 2000, 20036-5831.

23. Sugar and sweeteners yearbook, Economical Research Service, U.S. Department of Agricultural Washington, D.C. 2001, 20036-5831.

24. Sugar and Sweeteners Outlook/SSS-243/May 31, 2005, Economic Research Service, USDA: 3.

25. Tetu T, Sangwan RS, Sangwan-Norreel BS, Hormonal control of organogenesis and somatic embryogenesis in *Beta vulgaris* callus. J Exp Bot 1987, 38, 506–517.

26. Zilkah S, Faingersh E, Rotbaum, Dvorkin R, Eilam T, Anikster Y, Tissue culture of the alternate host of wheat, rye and oat rusts and their response to *in vitro* inoculation with rust pathogens. Plant Cell, Tissue and Organ Culture. 1999, 59(3), 209-215.

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