Final Report on Major Research Project

Project Tenure – Feb 2009 – July 2013

UGC Reference No: F. No. 37-1/2009 (MS) (SR)

Production of synthetic seeds in *Agave sisalana* Perr. Ex. Englem. for reforestation in the hilly barren land

A Major Research Project
Sanction By
University Grants Commission
Bahadur Shah zafar Marg
New Delhi -110 002

Principal investigator
Dr. T. D. Nikam Professor

Department of Botany
University of Pune
Pune – 411 007

DECLARATION

I hereby declared that the work presented under the Major Research project entitled **"Production of synthetic seeds in *Agave sisalana* perr. Ex. Englem. for reforestation in hilly barren land. "** is the outcome of my study carried out during the year Feb 2009 – July 2013. The work presented in the report is original and carried out according to the plan in the proposal and guidelines of the University Grant Commission.

Place: Pune **Dr. T. D. Nikam** **Professor**

Date:

ACKNOWLEDEGEMENT

Foremost, I would like to express my deep sense of gratitude to university grant commission, New Delhi for sanctioning the major research project and providing financial support for completion of project work.

I take immense pleasure in thanking the authorities of Savitribai Phule Pune University and Department of Botany for their co-operation and permitting me to carry out project work.

My sincere thanks go to all my colleagues and research scholar in the Department of Botany, University of Pune and administrative staff of Savitribai Phule Pune University, Pune for extending their co-operation.

I express my sincere thanks to librarian and staff of the Jaykar Library, Savitribai Phule Pune University; Departmental library, Department of Botany, Savitribai Phule Pune University, Pune; National Chemical Laboratory and Agharkar Research Institute, Pune for providing the facility for literature survey.

The Junior Research Fellow appointed under this project Miss. K.V. Mulye and Miss. S.R. Hanchate has worked sincerely. I use this occasion to express my deep sense of gratitude to them.

The endless list of people who have wholeheartedly helped us shall remain in our heart if not on paper.

T. D. Nikam

Dr. T .D. Nikam Professor,
Department of Botany,
Savitribai Phule Pune University,
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CONTENT

Table Content

Figure content

Annexure- III

Final report of the work done on the major research project

1. Project report No. Final

2. UGC Reference No. & Date: F. No. 37-468/2009 (SR) dated 11/01/2010

3. Period of report: from Feb 2010 to July 2013

4. Title of the Project: Production of synthetic seeds in *Agave sisalana* Perr. Ex. Englem. for reforestation in the hilly barren land

5. (a) Name of the Principal Investigator: Dr. T. D. Nikam

(b) Department and University where the project has undertaken: Department of Botany, University of Pune, Pune – 411 007.

6. Effective date of starting of the project: 01/02/2010

7. Grant approved and expenditure incurred during the period of the project:

a. Total amount approved Rs. **8,91,800/-**

b. Total expenditure Rs. 9, 31,221/- **(Details as: expenditure carried out Rs. 6,17,621+ payment to be made as fellowship arrears is Rs. 2,10,000/- and as a HRA Rs. 1,03,600/- under revised fellowship from 1/4/2010. {Ref.:UGC circular for revised fellowship** F-2-2/2011(SAP-II) dated Dec. 20110}

c. Report of the work done: (Please attach a separate sheet): Separate sheet is attached

Report of the work done

i. Brief Objectives of the Project:

1. Establishment of callus and cell culture.
2. Optimization of multiple shoot regeneration and somatic embryogenesis protocol.
3. Hardening and transfer of plantlets to field conditions.
4. To prepare the protocol for synthetic seeds and plantation of propagules.

ii. Work done so far and results achieved and publications, if any, resulting from the work:

Introduction

Indiscriminate cutting of trees as a result of urbanization, industrialization, mining operations and the use of wood for domestic and other purposes has caused heavy depletion of forest in India. At present 110 million hectors of wasteland exists in the country. In the state of Maharashtra nearly five million hectors is barren land. It is mostly in the hilly and mountain region. In most of the barren land only grass exists in the rainy season. In the next season dried grass biomass is lost due to wind and surface run-off water and the soil gets exposed. Naturally developed or planted seedlings are also lost because of forest fire, grazing animals and mainly due to the non-availability of water in the summer season. Deforestation leads to the loss of several million tones of valuable soil and minerals. It also prevents the percolation and holding of water in the soil. Underground sources of water that were present earlier dry later and several hundreds of villages face the problem of drinking water in the summer season. Formation of wasteland continues at quite a faster rate.

Although several efforts have been made continuously to develop the wastelands and their utilization for betterment of man and wildlife, the progress is too slow in this regard. The major cause for this is the non-availability of enough water in the summer season and good soil for growth of the plants. Irrigating all the planted seedlings is impractical.

The *Agave sisalana* Perr.ex.Engelm is a monocot, having importance being the source of about 90 % hard fibres are obtained from its leaf. The fibres are used for preparation of cordage and also pulped for making the paper(Mclaughlin and Susan, 1991). The plant is highly resistance to dry conditions and drought. The plant produce leaves continuously for the period of 8-30 years and flowers only once in life time and rarely or no seed setting occurs (Anonymous, 1980). On the other hand, the vegetative propagation is also to slow.

Agave plants are perennial xerophytes growing gregariously. Rootstock is rhizome, a short stem with leaves usually crowned radially on it, forming massive rosettes. Leaves are long broad, thick, fleshy, ensiform, prickly at margins and sharp-pointed. Each adult plant, under favorable conditions, can form few stolons every year, which produce new plants and spread in the surrounding area. This results in the formation of network of the roots covering a large area in the soil, which helps in avoiding the soil erosion. They also indirectly support and protect the other plants growing nearby. Most of the *Agave* plants rarely set the seeds and are conventionally

propagated by bulbils, which arise from the auxiliary meristems on the inflorescence after flowering, which occurs only after approximately 8 to 30 years of the vegetative growth.

Synthetic seed technology uses the encapsulation of *in vitro* propagules viz., somatic embryos, axillary buds, shoot apices, micro tubers, cormlets, bulbs, etc. for the preparation of the functional seeds. Implementation of this technology requires manipulation of embyogenic and other *in vitro* culture systems to obtain the viable material for encapsulation and successful conversion into plants. Although commercial feasibilities are being worked out, this technology offers great promise for use in cloning of elite germplasm of *Agave sisalana.*

To bring the barren hilly sloppy land under vegetation cover and also to check the soil erosion, plantation of *Agave sisalana* is one of the cost effective and guaranteed method. The planted *Agave* will also become a sustainable source of commercial hard fiber and pulp for paper industry.

The proposed research project "**Development of synthetic seeds in *Agave sisalana* and their application in the hilly barren land**" aims at generating a large number of suitable planting propagules and transforming them into the plantlets in the natural habitats.

WORK PLAN

The present work has been divided into two parts. The first part includes collection of plant material, establishment of callus culture, shoot regeneration and hardening of the plantlets. Second part includes induction of somatic embryogenesis, preparation of protocol for synthetic seed production and hardening of the plantlets from synthetic seeds.

Methodology

Source of explants

Stolons derived young plants and bulbils of *A. sisalana* were collected in the month of September and November 2010 from the regions of Ahmednagar, Nashik, Aurangabad, Kolhapur and Sangali districts of Maharashtra. The bulbils and stolon derived plantlets were established in pots at department of Botany, Savitribai Phule Pune University and used as source of explants.

Surface sterilization of explants and culture establishment

The stem and leaf explants were treated with 0.1% (w/v) mercuric chloride solution for 10 min. Then washed wash with sterile distilled water. The explants were inoculated on medium enriched with 4.44 µM BAP. All the cultures were incubated in a laboratory set at 16 hour light/8 hour dark photoperiod , 25 ± 2°C temperature and about 70% relative humidity.

***In vitro* shoot multiplication**

After one month of culture initiation, well established shoot cultures were transferred to different concentrations (0.0, 0.44, 2.26, 4.5, 6.67, 9, 18 or 27 µM) of cytokinins (BAP, KIN, Zeatin, TDZ or 2iP) to study their effect on shoot multiplication. The cultures were maintained under controlled condition as mentioned above.

Antibiotics and prevention of bacterial growth in shoot culture

Antibiotics like Plant Preservative Material (PPM), Streptomycin or Gentamycin were added in the medium to minimize the rate of contamination. Antibiotics were added as follows:

Plant Preservative Material (PPM): It is available in liquid form and can be added before or after autoclaving. In the present study 0.05, 0.06, 0.07, 0.08, 0.09 and 0.1% of PPM was used to control the rate of bacterial contamination.

Streptomycin: It is available in powder form. 1.0 gram streptomycin powder dissolved in 5.0 ml of sterile distills water. It is added aseptically to the autoclaved media at 50-60^{O}C in the laminar air flow. In the present investigation, 0.53, 0.67, 0.8, 0.93, 1, 1.2 or 1.3 mg ml^{-1} of streptomycin was used to control the rate of bacterial contamination.

Gentamycin: It is available in liquid form and can be added before or after autoclaving. In present investigation, 8, 16, 21 and 42 µg ml^{-1} of Gentamycin was used to control the rate of bacterial contamination.

4.5. Rooting of shoots

The in vitro raised shoots were subjected to root induction on medium consisting various concentrations (0.0, 0.27, 0.54, 1.07, 2.15 µM) of auxins (NAA or IAA). Single shoot was excised from the group of multiple shoots and was inoculated in culture tube containing above mentioned types and concentrations of auxin. Cultures were maintained for one month under the same culture condition which was maintained for the shoots initiation and multiplication.

4.6.Plantlets in field conditions

The plantlets were transferred to pots containing equal proportion of sand and soil. The pots were covered with transparent sheet for two weeks and maintained at culture room conditions. In third week, pots were subsequently exposed to reduced relative humidity and gradually increasing light intensity in the greenhouse. After two month, well hardened plants were then transferred in big plastic bags (25 x 40 cm) and exposed to field conditions for further growth.

4.7.Establishment of callus and cell culture

The stem and leaf explants prepared in the form circular disc and cultured on medium enriched by2,4-D(1.13 µM) together with BAP(4.5 µM). Approximately 200 mg calluses were transferred to medium fortified with 2,4-D, NAA or IAA (1.13, 1.36, 2.26 and 4.5 µM) together with BAP, TDZ and KIN (2.26 and 4.5 µM). The average fresh weight of the callus was determined. The callus from each treatment was dried at $60^{O}C$ until the callus reached a constant weight. The fresh and dry weight of callus was used to determined growth. The moisture content was determined by following formula:

% Moisture content = [(FW- DW)/ FW] x 100

The callus obtained on 2,4-D and BAP were hard and compact. Therefore, to increase the friability of callus for establishment of cell culture, various concentrations (200, 250, 300 and 350 mg l^{-1}) of KH_2PO_4 were added in MS liquid medium amended with same above mentioned compositions of hormone.

4.8.Morphogenic responses of callus

Morphogenesis in callus was observed on medium consisting permutation and combination of auxins and cytokinins (mentioned in table)

4.9.Induction of somatic embryos

Callus maintained by repeated sub-culturing over a period of six months was used for induction of somatic embryogenesis. The green healthy calluses were inoculated on medium consisting 2,4-D or NAA alone and together with BAP, KIN and TDZ.

4.10. Germination of somatic embryo and acclimatization of plantlet

The somatic embryo at globular stage were transferred on media consisting KIN and observed for maturation and germination.

4.11.Encapsulation of somatic embryo and shoots

Somatic embryos at globular stage and shoot bases were suspended in MS (except $CaCl_2$) consisting various concentrations (2, 4, or 6%) of sodium alginate and the suspension is dropped into the different concentrations of (50, 100 or 150 mM) $CaCl_2.2H_2O$ solution. For proper bead formation, bead containing flask was place on a shaker at 70-90 rpm for 25 min. Followed by washing with sterile DW and blot using sterilized filter paper. The operation were carried out under sterile conditions. Finally the synthetic seeds germination was observed on medium consisting cytokinins (BAP or KIN) together with auxin (NAA). The germinated embryos were maintained at culture room conditions.

4.12.Hardening and acclimatization of synthetic seed derived plantlets

The preliminary treatments were similar as used for transfer of normal in vitro raised plantlets. The pots were covered with transparent sheet and maintained for two weeks in culture room. In third week, pots were subsequently exposed to reduced relative humidity and gradually increasing light intensity in the greenhouse. After two month, well hardened plants were then transferred in big plastic bags (25 x 40 cm) and exposed to field conditions for further growth.

4.13.PEG and NaCl treatment to hyperhydric shoots

After several cycles of subcultures of shoots of *A. sisalana* on highest shoot multiplication media were showing typical hyperhydricity symptoms. These hyperhydric shoots were grown on medium consisting with or without NaCl and PEG 6000 (0, 0.1, 0.2, 0.3, 0.4, or 0.5 %). The cultures were observed for recovery of hyperhydricity symptoms. The cultures were then analyzed for changes in physiology, biochemistry and anatomy.

4.14.Rooting and hardening of hyperhydric recovered shoots

After removal of hyperhydric symptoms, the plantlets were hardened and transferred to the natural conditions.

4.15.Study of physiological and biochemical parameters

4.15.1. Water content in tissue

The water content in tissue was calculated using FW and DW of the samples and represented in the form of TWC %. TWC% = [(FW- DW)/ FW] x 100

4.15.2. REL:

The method of Sullivan (1972) was used for knowing the damage to the membrane and it was calculated in terms of REL. REL = (EC1/ EC2) x100.

4.15.3. Lipid peroxidation

The TBA method of Heath and Packer (1968) was used to find the formation of malondialdehyde (MDA). The lipid peroxidation was represented in terms of µmol of MDA formed per gram fresh weight (EC =155 mM cm^{-1}).

4.15.4. Total soluble sugars (TSS)

Watanabe et al. (2000) method was followed for determination of TSS. OD of the sample was taken at 620 nm. D- Glucose was used as standard sugar. TSS of samples was calculated and express as mg g^{-1} FW.

4.16. Anatomical and ultra-structural analysis

4.16.1. Stomatal characteristics

Epidermal peel was prepared from normal (control and treated) and hyperhydric plantlets. The samples were observed at 100x and stomatal density was calculated. Stomatal index (SI) = [S/(E + S)] × 100, where S = number of stomata, and E = number of epidermal cells per unit leaf area (Salisbury 1927; Pompelli et al. 2010).

4.16.2. Size of the stomata

For the measurement of stomatal size, control and treated plant leaf peel sample was mounted in 10% (v/v) glycerine on glass slide and observed under compound microscope (Olympus OIC–612086, Japan). Microscope was first calibrated using stage (0.01mm) and ocular scale. After calibration of microscope, the prepared slide was kept under it and measuring of stomata size was carried out at 400X magnification.

4.16.3. Light microscopy

Leaf samples were collected from normal, hyperhydric and treated hyperhydric shoots. The sections stained with safranin (1%w/v) and placed on slide in 50% glycerine, followed by sealing with transparent nail pent. The slides were then observed under compound microscope (Olympus OIC–612086, Japan) at 100X. The size difference

between the cells of normal and hyperhydric samples were measured at 100X magnification by using micrometry technique.

4.16.4. SEM-observations

The samples (0.5 cm^2) were fixed in glutaraldehyde(2.5%). Then dehydration was carried out by placing in increasing grades of ethanol(10 – 100%) for 60 min in each grade. Leaf surface scanning were carried out using analytical electron microscope (JEOL JSM-6360A, Japan).

5. Results:

5.1. Establishment of stock plants and Aseptic cultures

Agave sisalana plants were collected from different localities of state of Maharashtra and accession were grown in Botanic Garden. Stolon derived young plants and bulbils of *Agave sisalana* were procured from 12 different places of state of Maharashtra. The excised stem and apical explants after surface sterilization incubated on 4.5 µM BAP containing MS medium. Three bacterial colonies white, yellow and red coloured were appeared frequently in one to four months old cultures. These bacteria drastically affect the explant growth and shoot multiplication. The elimination of bacteria was achieved by using the treatments of three antibiotics namely; PPM^{TM}, Streptomycin and Gentamicin. The gentamicin 942 µg ml^{-1}) was highly effective to prevent the growth of these bacteria. The multiple shoots formation was not affected at this concentration of gentamicin.

5.2. Shoot regeneration

The explants from in *vitro* raised shoots were inoculated on media consisting of auxins and cytokinins, total of 284 permutations and combinations. The maximum frequency of explants responding to shoot regeneration (100 %) and number (8.7±0.12) of shoots per culture were recorded on MS consisting 9 µM BAP. The shoot tips explants were subculture on fresh parental medium for shoot multiplication over a period of two and half year.

5.3. Callus culture and cell culture

The good callus induction and proliferation observed on media consisting 2,4-D, IAA, NAA (1.13, 1.36, 2.26 and 4.5µM) together with BAP, TDZ and KIN (2.26 and 4.5 µM). The extensive callus formation was observed on medium incorporated with 2, 4-D

and BAP. 1.13 µM 2, 4-D and 4.5 µM BAP containing medium was superior for callus and cell culture. The callus was maintained in continuous growing conditions on subculture to parental medium at three weeks interval over a period of two and half year.

The results on influence of KH_2PO_4(200-350 mg l^{-1}) on friability of callus is summarized in Fig. 11- a-b. Inclusion of KH_2PO_4 significantly increased the friability of callus (Fig. 11- b). Maximum friability of callus was obtained at 350 mg l^{-1} of KH_2PO_4. Whereas, KH_2PO_4 used at 200 - 300 mg l^{-1} concentrations was not found suitable for inducing friability in callus.

5.4. Somatic embryogenesis and embryo germination

The selected media were used for maintenance of actively growing callus masses. The selected media were fortified with casein hydrolysate, yeast extract, potassium nitrate and amino acids. Maximum number (24.6 ± 0.04) of somatic embryos per culture was recorded on 2, 4-D (2.26 µM) together with TDZ (1.13 µM) containing medium. The globular embryos from callus were subculture to MS basal medium resulted in germination and formation of complete plantlets.

From embryogenic calluses on medium fortified with 0.0, 0.1 and 0.2 mg/l KIN, the maturation of somatic embryos was achieved within 3 weeks. During subsequent fourth and fifth week, germinated embryos were developed into well rooted plantlets. The medium consisting 2,4-D(1.13 µM) + BAP(4.5 µM) was suitable for cell culture. Somatic embryogenesis was not observed in cell culture.

5.5. Rooting of shoots and hardening

The separated shoots were subjected for rooting on MS medium lacking auxins and consisting IAA, NAA or IBA. The shoots showed direct root formation within two weeks of incubation on NAA (0.54 µM) consisting medium. The plantlets were hardened and successfully grown to field condition with 98 % survival. The leaves attain the length of 20-30 cm after 4 months of transfer to field conditions. Embryo derived plantlets showed 100% survival in field after 2 weeks of acclimatization in controlled conditions. The plantlets were morphologically similar with the mother plants.

5.11.Rescue of hyperhydricity

Hyperhydricity is a severe problem in the micropropagation of *A. sisalana*, which affects shoot growth and multiplication process. It resulted up to 50-60% reduction in the

production of *A. sisalana* plants .Incorporation of various levels of NaCl and PEG were effective for rescue of hyperhydricity in the shoots. The medium containing 0.2% NaCl was found to be effective for complete recovery of hyperhydric shoots.

5.12.Influence of osmotic agent on pigment content

The observations on pigment (chl *a,* chl *b,* total chl and carotenoid) content in in vitro raised normal (control and treated) and hyperhydric are depicted in Table 11. The data revealed that pigment (chl *a,* chl *b,* total chl and carotenoid) content decline drastically in Hyperhydric shoots over the normal and osmotic agent treated shoots (Table 11). However, pigment (chl *a*, chl *b,* total chl and carotenoid) content was similar in normal and osmotic agent treated shoots (Table 11). The results revealed that the treatment of osmotic agent NaCl and PEG was effective for recovery of hyperhydric shoots.

5.13.Osmotic agent and oxidative

The observation on oxidative damage in terms of REL and MDA content in in vitro raised normal(control and treated) and hyperhydric, are depicted in Table 12. REL and MDA content of hyperhydric shoots was significantly higher (69.38%) in comparison to control as well as NaCl and PEG treated hyperhydric shoots (Table 12). However, the REL of both NaCl and PEG treated hyperhydric shoots showed lower value in comparison to control (Table 12). In contrast, MDA content of both the shoots was significantly lower or as par to control (Table 12). Therefore, REL and MDA content, a measure of oxidative damage also proved the recovery of hyperhydric shoots upon their exposure to NaCl and PEG and their phenotype as par to control.

5.14.Osmotic agent and accumulation of TSS

The results on accumulation of TSS in in vitro raised normal, hyperhydric, and reverted shoots with the exposure of NaCl and PEG are depicted in depicted in Table 12. Accumulation of TSS was found to be significantly lower in hyperhydric shoots in comparison to control and the hyperhydric shoots exposed to NaCl and PEG (Table 12), which indicates lower tolerance response to osmotic stress. However, reverted shoots with the exposure of NaCl and PEG showed almost similar accumulation of TSS when compared with control (Table 12).

5.15. Influence of NaCl and PEG on stomatal, anatomical and ultrastructural characteristics

5.15.1. Influence of NaCl and PEG on stomatal characteristics

The results on stomatal observations of in vitro raised normal, hyperhydric, and reverted shoots with the exposure of NaCl and PEG are depicted in Table 13. Microscopic qualitative and quantitative analysis of the stomata revealed that, in hyperhydric shoots stomata were larger compare to control, and reverted shoots with the exposure of NaCl and PEG (Table 13). However, stomata observed in control, NaCl and PEG treated shoots revealed spherical shape with almost similar length and breadth (Table 13). At the same time, in hyperhydric shoots stomatal index and density were lower compare to normal, and reverted shoots with the exposure of NaCl and PEG (Table 13). Therefore, the incorporation of NaCl and PEG showed added effect on stomatal character.

5.15.2. Influence of NaCl and PEG on anatomical and ultrastructural observations

Light microscopic qualitative analysis revealed considerable differences in the leaf anatomy of in vitro raised normal, hyperhydric, and reverted shoots with the exposure of NaCl and PEG plant samples depicted in Fig. 9- a-f. In control, leaf had continuous epidermis with uniform epidermal cells followed by mesophyll cells which were homogeneous and very dense with abundant chloroplast. Mesophyll was embedded with number of scattered conjoint, collateral vascular bundles of various sizes (Fig. 9- a, d). However, in hyperhydric plant material, the epidermis was not continuous and the cells were uneven and large in size. The development of chlorenchyma and lignified cells (xylem) was very poor (Fig. 9- b, c, e, and f).

SEM analysis of leaf surface suggest that the stomatal guard cells were superficial in position similar to normal epidermal cells. While in control, the stomatal guard cells were sunken in position (Fig. 10- a, c). However, in NaCl treated hyperhydric plant material, stomata were sunken and in PEG treated sample, stomata were still found in raised position (Fig. 10- e, g). Micrographs of control leaf showed parallel arrangement of epicuticular wax platelets (Fig. 10- a). However in hyperhydric plant material, epicuticular wax plug in dendrite form (Fig. 10- d). In NaCl treated leaf sample, stomata showed formation of platelets of epicuticular wax in striking form around it similar to the

control stomata (Fig. 10- f); whereas, in PEG treated sample, stomata showed absence of Platelets of epicuticular wax and epicuticular stomatal plug(Fig. 10- h).

5.16.Synthetic seeds

The sodium alginate together with $CaCl_2.2H_2O$ was used for encapsulation of somatic embryos and shoots. The incorporation of sodium alginate (4%) together with $CaCl_2.2H_2O$ (100 mM) was superior for obtaining the uniform beads. Among the shoots and embryo derived synthetic seeds, maximum germination (95%) was obtained in shoots derived synthetic seeds on medium consisting BAP (4.5 µM) and NAA (0.54 µM).

5.17.Acclimatization of plantlets in natural field

Well rooted normal and synthetic seed derived plantlets were subjected to acclimatization in field condition. Over five hundred of plantlets successfully harden and transferred to field condition. Shoot derived synthetic seed plantlets show 95 % survival whereas, embryo derived synthetic seed plantlet shows 85 % survival. The plantlets shifted to natural environmental conditions were phenotypically equivalent to the mother plants.

Principal Investigator **Head**

Registrar

Annexure- VIII

FINAL REPORT OF THE WORK DONE ON THE PROJECT

1. **NAME AND ADDRESS OF THE PRINCIPAL INVESTIGATOR:** Dr. T.D. Nikam, 'Professor, Department of Botany, University of Pune, Pune 411 007.

2. **NAME AND ADDRESS OF THE INSTITUTION:** Department of Botany, University of Pune, Pune- 411007

3. **UGC APPROVAL NO. AND DATE:**F. No. 37-468/2009 (SR) dated 11/01/2010

4. **DATE OF IMPLEMENTATION:** 01/02/2010

5. **TENURE OF THE PROJECT :** 3 years from 01/02/2010 to 31/01/2013

6. **TOTAL GRANT ALLOCATED :** 8,91,800/-

7. **TOTAL GRANT RECEIVED:** 5,92,800/-

8. **FINAL EXPENDITURE:** 6,17,621/-
   (Amount to be receive from the UGC Rs. 3, 13,600/-)

9. **TITLE OF THE PROJECT:** "Production of synthetic seeds in *Agave sisalana* Perr. Ex. Englem. for reforestation in the hilly barren land"

10. **OBJECTIVES OF THE PROJECT:**

    1. Establishment of callus and cell culture.

    2. Optimization of multiple shoot regeneration and somatic embryogenesis protocol.

    3. Hardening and transfer of plantlets to field conditions.

    4. To prepare the protocol for synthetic seeds and plantation of propagules.

11. WHETHER OBJECTIVES WERE ACHIEVED(GIVE DETAILS): Yes

All the proposed objectives of the present study were attended. The protocol for establishment of callus and cell culture, multiple shoot regeneration by direct and indirect method, protocol for synthetic seeds and plantation of propagules and hardening and transfer of plantlets to field conditions was successfully achieved. The best in vitro propagation system was developed to satisfy the demand of propagules for plantation of *Agave sisalana*.

12. ACHIEVEMENTS FROM THE PROJECT:

1. *Agave sisalana* plants were collected from different localities of state of Maharashtra and accession were established at Botanic Garden, Department of Botany, Savitribai Phule Pune University.

2. On application of different nutrient media with auxins and cytokinins the protocol for callus and cell cultures was established from stem and leaf explants. The best medium for callus and cell culture was MS + 2, 4-D (1.13 µM) + BAP (4.5 µM).

3. The healthy green callus was maintained by sub culturing over a period of two years.

4. Total near about 284 permutations and combinations were tried for shoot induction. Best response for shoot regeneration was observed from stem explants. About 9 to15 shoots per stem explants were produced on MS +9 µM BAP. Regenerated shoots showed the hyperhydricity which was overcome by the treatment of NaCl.

5. The root induction was best on medium lacking auxins and supplemented with NAA.

6. About 95% of the plantlets were showed survival and good growth in field conditions. The leaves attain the length of 20-30 cm after 4 months of transfer to field conditions. The plantlets were morphologically similar with the mother plants.

7. Among the different media tested, the best media for induction of somatic embryogenesis in callus was 2.26 µM 2,4-D + 1.13 µM TDZ.

8. The globular embryos from callus were subculture to MS basal medium resulted in germination and formation of roots and shoot within 8 weeks of culture.

9. The synthetic coating of sodium alginate (4%) and calcium chloride (100 mM) was supportive for formation of uniform beads, coat hard enough for handling and significantly higher frequency of synthetic seed germination. From synthetic seeds, maximum about 85-95% survival of plantlets was achieved.

10. MS consisting 2, 4-D (1.13 µM) and BAP (4.5 µM) was suitable for callus and cell culture; however, none of the media tested were found effective for induction of somatic embryo.

11 From synthetic seeds, maximum about 85-95% survival of plantlets was achieved. The plantltes were phonetically similar to the mother plants.

12. The developed protocol can be used for preparation of large number of propagules for plantation in dry barren land.

13. SUMMARY OFTHE FINDING (GIVE DETAILS IN 500 WORDS):

Agave sisalana is one of the best plant adapted dry climate of the tropical and subtropical region. The leaves are the source of commercial hard fibres, besides the plant parts are also used for medicnal purpose. The plant can grow well on sandy rocky soil and on sloppy hilly land area. It is good candidate for re-establishment of forest cover on hilly barren land. However, natural propagation is not sufficient to satisfy the demand of plantation. Therefore, in the present investigation the attempts were made to establish the best in-vitro propagation protocol through organogenesis and somatic embryogenesis. Further to develop the synthetic seeds production protocol and transfer of plantlets to field conditions. To raise this work, best accession of A.siasalana were collected from different locations in the state of Maharashtra and established as a source of explants. The cultures were established using stem, leaf and bulbil explants on MS medium fortified with BAP, KIN, TDZ, ZEATIN, IAA, NAA and 2,4-D. The explants, stem explants was best responsive for multiple shoot formation. Among the media used, MS +9 µM BAP medium was most suitable for multiple shoot formation (9 to15 shoots per explants). Auxins IAA, NAA and IBA were used for induction of rooting; medium containing 0.54 µM NAA induce direct rooting to the shoot. MS consisting 2, 4-D (1.13 µM) and BAP (4.5 µM) was suitable for callus and cell culture; on this medium callus and cell culture was maintained over period of two years. Various media were used for induction of somatic embryogenesis, media consisting 2.26 µM 2,4-D + 1.13 µM TDZ was effective for induction and growth of somatic embryo. The shoots after partial removal of

leaf and somatic embryos at globular to maturation stage were used for synthetic seed production by application of coating of sodium alginate and calcium chloride. The synthetic coating of sodium alginate (4%) and calcium chloride (100 mM) was supportive for formation of uniform beads, coat hard enough for handling and significantly higher frequency of synthetic seed germination. From synthetic seeds, maximum about 85-95% survival of plantlets was achieved. The plantltes were phonetically similar to the mother plants. The developed protocol can be used for preparation of large number of propagules for plantation in dry barren land..

14. **CONTRIBUTION TO THE SOCIETY (GIVE DETAILS):** On application of the developed protocol, it is possible to bring the hilly barren land under plantation which will help in soil and water conservation, availability of commercial hard fibers and important metabolites hecogenin and Tigogenin. This will help in generation of employment and improvement of economy

15. **WHETHER ANY Ph. D. ENROLLED/PRODUCED OUT OF THE PROJECTS:** M.Phil degree of the Pune University is awarded with Grade A to Miss. Ketki Vilas Mulye on 20 may 2013.

16. **NO. OF PUBLICATIONS OUT OF THE PROJECT:** One Publications is under communication and one under preparation

1. Control of hyperhydricity in shoot cultures of *Agave sisalana* Perr. Ex. Englem. Plant cell, tissue and organ culture. Springer

2. Enhancement of hecogenin and tigogenin in cell cultures of Agave *sisalana* Perr. Ex. Englem using organic supplements and elicitors. Applied microbiology and biotechnology. Springer

Principal Investigator **Head**

Registrar

Table 1: Effect of antibiotics and PPM on control of bacteria in shoot cultures of *A. sisalana*.

Antibiotic/Sterilizing agent(PPM)	Prevention of bacterial growth	Prevention of bacterial growth in cultures (%)	Colour of bacterial contaminant
PPM^{TM} (%)			
0.05	-	-	-
0.06	-	-	-
0.07	+	35^{d}	Yellow
0.08	+	50^{c}	Yellow
0.09	++	65^{b}	Yellow
0.1	++	75^{a}	Yellow
Streptomycin (mg/l)			
0.53	+	40^{d}	white and yellow
0.67	+	50^{c}	white and yellow
0.8	++	70^{b}	white and yellow
0.93	++	70^{b}	white and yellow
1.0	++	85^{a}	white and yellow
1.2	++	90^{a}	white and yellow
1.3	++	90^{a}	white and yellow
Gentamicin(µg/ml)			
8	+	25^{d}	White, yellow and red
16	+	50^{c}	White, yellow and red
21	++	90^{b}	White, yellow and red
42	+++	100^{a}	White, yellow and red

Mean followed by same letter are not significantly different at *5%* level by DMRT

The sign indicates "–": No; "+": good; "++": better and "+++": best response for removal of bacteria in the medium.

Table 2: Effect of BAP, KIN and TDZ on in vitro shoot multiplication in *A. sisalana*.

PGRs	Concentrations (µM)	Number of multiple shoots per explant	% of explants responded to multiplication
MS basal	-	-	-
BAP	0.44	-	-
	2.26	1.3±0.12^{c}	50^{d}
	4.5	1.46±0.12^{c}	80^{c}
	6.67	2.9±0.1^{b}	95^{b}
	9	8.7 ±0.12^{a}	100^{a}
	18	1.02±0.11^{c}	35^{e}
	27	-	-
KIN	0.44	-	
	2.26	1.03±0.24^{c}	35^{c}
	4.5	2.2±0.40^{b}	60^{b}
	6.67	3.3±0.20^{a}	85^{a}
	9	2.23±0.12^{b}	65^{b}
	18	-	-
	27	-	-
Zeatin	0.44	-	-
	2.26	1.1±0.12^{b}	20^{c}
	4.5	1.9±0.33^{a}	35^{b}
	6.67	1.7±0.14^{a}	55^{a}
	8.87	-	-
	18	-	-
	27	-	-

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 3: Effect of 2,4-D in combination with BAP or KIN on callus induction in shoot and leaf explants of *A. sisalana*

MS + PGRs (µM)			Days required to explants for callus induction		% explants responding for callus induction	
2,4-D	BAP	KIN	shoot	leaf	shoot	leaf
1.13	2.26		10^{a}	19^{a}	65^{c}	20^{c}
	4.5		7^{c}	15^{b}	100^{a}	20^{c}
1.36	2.26		9^{b}	16^{b}	55^{d}	35^{c}
	4.5		7^{c}	12^{d}	50^{d}	85^{b}
2.26	2.26		9^{b}	12^{d}	50^{d}	95^{a}
	4.5		9^{b}	14^{c}	95^{b}	15^{e}
4.5	2.26		10^{a}	15^{b}	60^{c}	25^{d}
	4.5		9^{b}	-	40^{e}	-
1.13		2.26	15^{a}	-	35^{d}	-
		4.5	13^{b}	-	50^{c}	-
1.36		2.26	12^{b}	-	80^{a}	-
		4.5	13^{b}	-	65^{b}	-
2.26		2.26	15^{a}	-	55^{c}	-
		4.5	15^{a}	19^{a}	50^{c}	65^{a}
4.52		2.26	-	19^{a}	-	55^{b}
		4.5	-	-	-	-

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 4: Effect 2,4-D together with BAP, KIN and TDZ on somatic embryogenesis in *A. sisalana*.

Plant growth regulators (µM)				% embryonic cultures	Average no. of somatic embryos per culture
2,4-D	BAP	KIN	TDZ		
1.13	1.13			-	-
	2.26			35^{f}	6.5 ± 0.02^{e}
	4.5			85^{b}	10.9 ± 0.02^{b}
	6.67			65^{d}	9.0 ± 0.07^{c}
	9			55^{e}	3.5 ± 0.02^{h}
2.26	1.13			25^{g}	5.6 ± 0.03^{f}
	2.26			-	-
	4.5			95^{a}	12.9 ± 0.02^{a}
	6.67			75^{c}	8.3 ± 0.05^{d}
	9			50^{e}	4.5 ± 0.03^{g}
1.13		1.13		20	1.4 ± 0.03
		2.26		45^{a}	4.2 ± 0.01^{b}
		4.5		15^{e}	2.2 ± 0.02^{e}
		6.67		10^{f}	3.3 ± 0.02^{c}
		9		15^{e}	1.1 ± 0.03^{g}
2.26		1.13		30^{c}	2.2 ± 0.03^{e}
		2.26		15^{e}	1.6 ± 0.02^{f}
		4.5		10^{f}	1.2 ± 0.03^{g}
		6.67		35^{b}	5.3 ± 0.02^{a}
		9		25^{d}	2.5 ± 0.02^{d}
1.13			1.13	-	-
			2.26	25^{e}	4.3 ± 0.2^{e}
			4.5	45^{c}	8.9 ± 0.01^{b}
			6.67	20^{e}	1.6 ± 0.1^{g}
			9	-	-
2.26			1.13	95^{a}	24.6 ± 0.04^{a}
			2.26	55^{b}	6.3 ± 0.02^{c}
			4.5	45^{c}	4.4 ± 0.02^{d}
			6.67	35^{d}	2.6 ± 0.04^{f}
			9	-	-

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 5: Effect of KN on somatic embryo germination and plantlet formation of *A. sisalana*

MS + KIN mg/l	Number of somatic embryo germinated	Germination(%)	Plantlet formation(%)
0.0	4.5 ± 0.97^{ab}	45.7 ± 9.75^{ab}	24.2 ± 9.75^{a}
0.1	7.5 ± 1.39^{a}	75.7 ± 13.97^{a}	65.7 ± 13.9^{b}
0.2	4.5 ± 0.97^{ab}	45.7 ± 9.75^{ab}	14.2 ± 5.34^{a}

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 6 : Induction of rooting in in vitro raised shoots of *A. sisalana*.

PGRs	Concentrations (µM)	Average number of roots /shoot	Average length of root (cm)	Percent shoot responded to rooting
control	0.0	-	-	-
NAA	0.27	4.6 ± 0.13^{b}	8.3 ± 0.02^{b}	85^{b}
	0.54	7.15 ± 0.07^{a}	8.9 ± 0.01^{a}	100^{a}
	1.07	3.4 ± 0.19^{c}	6.9 ± 0.03^{c}	65^{c}
	2.15	3.17 ± 0.03^{c}	5.5 ± 0.01^{d}	40^{d}
IAA	0.27	3.9 ± 0.07^{c}	3.4 ± 0.02^{d}	45^{c}
	0.54	4.4 ± 0.07^{b}	4.3 ± 0.1^{c}	70^{b}
	1.07	4.6 ± 0.1^{a}	5.34 ± 0.07^{b}	85^{a}
	2.15	3.6 ± 0.09^{d}	5.58 ± 0.01^{a}	35^{d}

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 7: Influence of osmotic agent on recovery of hyperhydric shoots of *A. sisalana*

Osmotic agent	Concentration (%)	Recovery of average number of plantlets	percent recovery
NaCl	0.1	9^{b}	45^{b}
	0.2	16.7^{a}	85^{a}
	0.3	7.8^{c}	38.3^{c}
	0.4	4.3^{d}	21.65^{d}
	0.5	0^{e}	0^{e}
PEG 6000	0.1	11.9^{a}	58.3^{a}
	0.2	7.6^{b}	38^{b}
	0.3	5.3^{c}	26.65^{c}
	0.4	2.6^{d}	11.7^{d}
	0.5	1^{e}	5^{e}

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 8 : Influence of osmotic agent on FW, DW and RWC in in vitro raised shoots of *A. sisalana*

in vitro raised shoot type	FW (gm)	DW (gm)	RWC (%)
Normal (control)	0.23 ± 0.05^{b}	0.029 ± 0.06^{c}	88.26^{b}
Hyperhydric	0.53 ± 0.08^{a}	0.016 ± 0.01^{d}	96.95^{a}
Reverted to normal type on 0.1% NaCl	0.19 ± 0.01^{c}	0.077 ± 0.05^{a}	60.61^{c}
Reverted to normal type on 0.2% PEG 6000	0.12 ± 0.01^{d}	0.050 ± 0.05^{b}	59.27^{c}

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 9: Influence of osmotic agent on Chl *a*, chl *b*, total chl and carotenoids content in in vitro raised shoots of *A. sisalana*

In vitro raised shootb type	Chl *a* (mg g^{-1} FW)	Chl *b* (mg g^{-1} FW)	Total chl (mg g^{-1} FW)	Total carotenoids (mg g^{-1} FW)
Normal shoots	0.039 ± 0.01^{a}	0.021 ± 0.003^{a}	0.060 ± 0.01^{a}	0.019 ± 0.001^{a}
Hyperhydric shoots	0.021 ± 0.01^{d}	0.011 ± 0.001^{b}	0.031 ± 0.002^{b}	0.012 ± 0.001^{b}
Reverted to normal type on 0.1% NaCl	0.035 ± 0.01^{b}	0.022 ± 0.002^{a}	0.056 ± 0.01^{a}	$0.014 \pm 0.001^{a,b}$
Reverted to normal type on 0.2% PEG 6000	0.031 ± 0.02^{c}	0.023 ± 0.001^{a}	0.053 ± 0.01^{a}	0.016 ± 0.002^{a}

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 10: Influence of osmotic agent on oxidative damage to membrane in control and hyperhydric shoot of *A. sisalana*.

In vitro raised shoot type	REL (%)	MDA (µmol g^{-1} FW)	TSS (mg g^{-1} FW)
Normal	32.84^{d}	8.46 ± 0.12^{b}	6.1 ± 0.15^{a}
Hyperhydric	69.38^{a}	12.27 ± 0.66^{a}	4.21 ± 0.26^{c}
Reverted to normal type on 0.1% NaCl	36.65^{c}	7.80 ± 0.21^{c}	5.2 ± 0.05^{b}
Reverted to normal type on 0.2% PEG 6000	45.40^{b}	6.66 ± 1.42^{d}	5.1 ± 0.13^{b}

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 11: Stomatal observations of in vitro raised normal, hyperhydric, and hyperhydric reverted shoots with the treatment of NaCl and PEG

In vitro raised shoot type	Stomatal characteristics			
	Breadth(um)	Breadth(um)	density / mm^2	index (%)
Normal	0.38 ± 0.02^{a}	0.41 ± 0.01^{b}	30 ± 1.78^{a}	7.2^{a}
Hyperhydric	0.35 ± 0.02^{b}	0.56 ± 0.03^{a}	11.58 ± 0.64^{c}	4.97^{c}
Reverted to normal type on 0.1% NaCl	0.38 ± 0.02^{a}	0.40 ± 0.01^{b}	16.84 ± 1.96^{b}	5.77^{b}
Reverted to normal type on 0.2% PEG 6000	0.38 ± 0.02^{a}	0.39 ± 0.01^{b}	15.27 ± 0.56^{b}	5.20^{b}

Mean followed by same letter are not significantly different at *5%* level by DMRT

Table 12: Effect BAP or KIN together with NAA on germination of synthetic seeds.

Synthetic seeds derived from	BAP (µM)	KIN (µM)	NAA (µM)	No. of Days for germination	Frequency of germination (%)
Shoot	0.0		0.0	-	-
	2.26		0.27	18^{c}	67^{c}
	4.5		0.54	10^{f}	95^{a}
	6.67		1.07	12^{e}	59^{d}
	9		2.15	15^{d}	43^{f}
		2.26	0.27	19^{b}	52^{e}
		4.5	0.54	19^{b}	66^{c}
		6.67	1.07	18^{c}	65^{c}
		9	2.15	20^{a}	43^{f}
Somatic embryo	0.0			-	-
	2.26		0.27	18^{c}	45^{f}
	4.5		0.54	19^{b}	56^{d}
	6.67		1.07	15^{d}	80^{b}
	9		2.15	17^{c}	65^{c}
		2.26	0.27	18^{c}	30^{h}
		4.5	0.54	19^{b}	38^{g}
		6.67	1.07	17^{c}	62^{c}
		9	2.15	18^{c}	50^{d}

Mean followed by same letter are not significantly different at *5%* level by DMRT

Fig. 1 Shoot multiplication

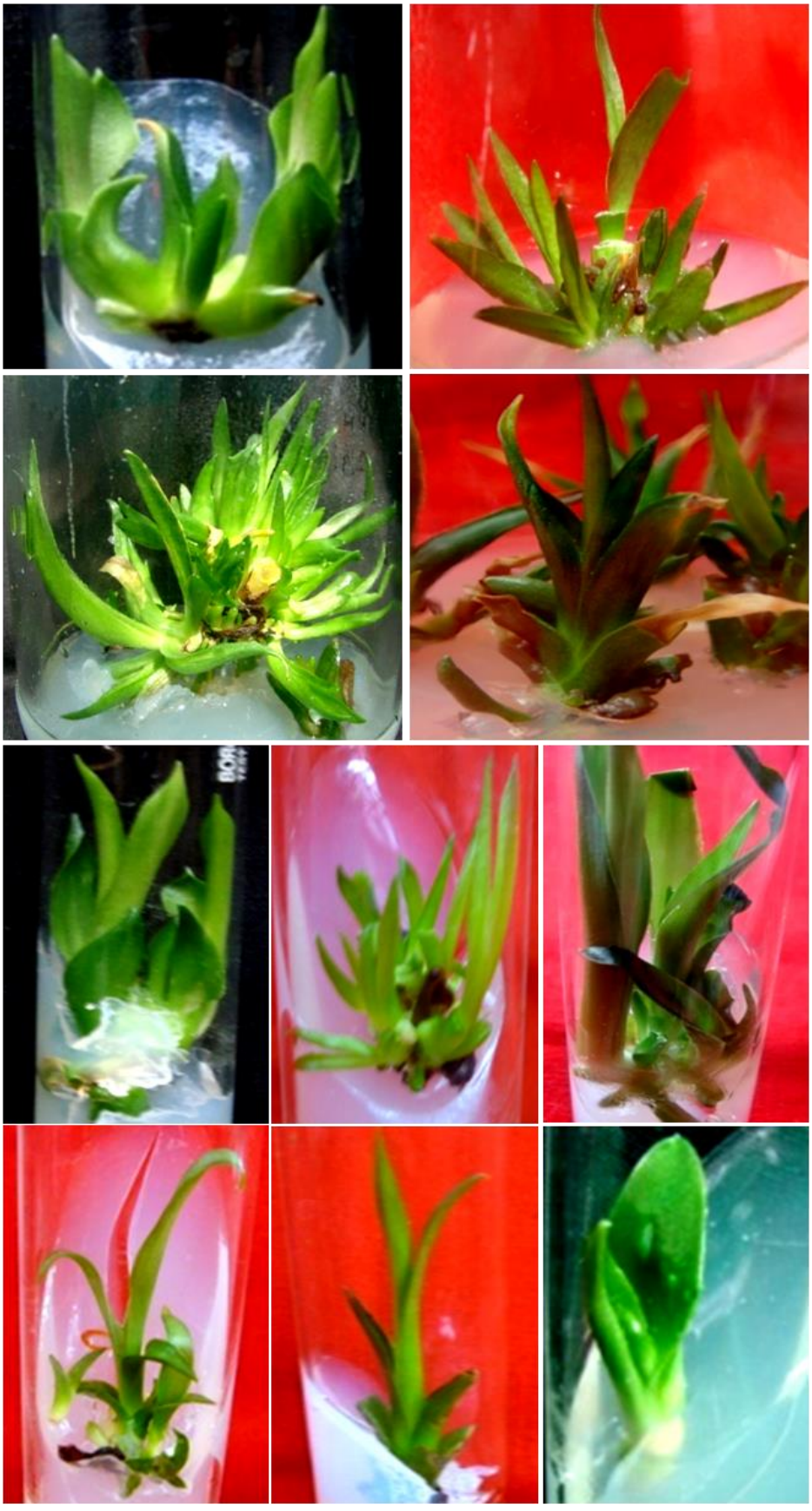

Fig. 2 Rooting of shoots

Fig.3

Shoot culture on large scale

Fig. 4

Hardening and acclimatization

Fig. 5

Acclimatization of plantlets

Fig. 6 Callus culture

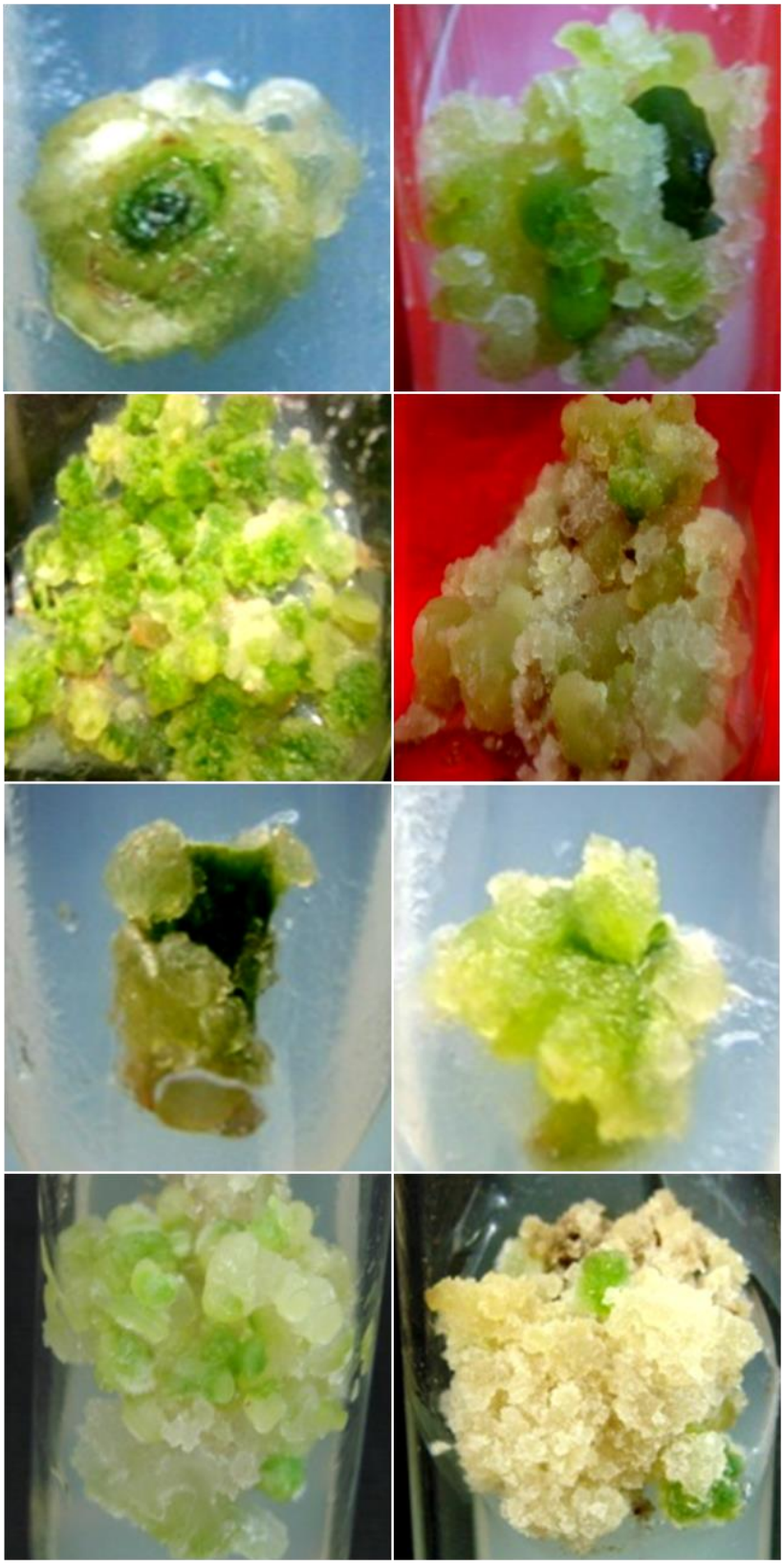

Fig. 7

Morphogenic responses of callus

Fig. 8

Somatic embryogenesis

Fig. 9 Hyperhydricity and recovered hyperhydric shoots

Production of synthetic seeds in Agave sisalana Perr. Ex. Englem. for reforestation in the hilly barren land

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