MINOR RESEARCH PROJECT REPORT

ESTABLISHING OPTIMUM *IN VITRO* CULTURE CONDITIONS FOR RAISING THE MORPHOGENIC CULTURES WITH HIGH REGENERATION POTENTIAL IN MAJOR MILLETS.

Submitted to

University Grants Commission (UGC)
Bhopal (MP)
By

Dr. Rajesh Singh Nirwan (PI)
(Department of Botany)

SETH MOTILAL (PG) COLLEGE,
College with Potential for Excellence by UGC
Conferred as Model College by the Government of Rajasthan
JHUNJHUNU-333001 (RAJASTHAN)

Seth Motilal (P.G.) College

College with Potential for Excellence by UGC
Conferred as Model College by the Government of Rajasthan
Jhunjhunu-333001 (Raj.)

☎ 01592-232326
232461, 238484

Ref. No. SMC/2017/..... Date:

To,

Joint Secretary
University Grants Commission
Central Regional Office
Tawa complex (Bittan market)
E-5 Erera Colony
Bhopal -462016 9MP)

Subject: Submission of final report and statement of expenditure of Minor Research Project entitled, "Establishing optimum *in vitro* culture conditions for raising the morphogenic cultures with high regeneration potential in major millets".

Ref: - MS-49/304044/XII/14-15/CRO Dated: 01May, 2015

Dear Sir,

This is with reference to above cited subject that I hereby want to submit the final report of Minor Research Project which was sanctioned to me by your letter no.MS- 49/304044/XII/14-15/CRO. Two copies of project report are being sent to you for your kind perusal. I do hope that you will find them in good order and acknowledge for the receipt of report. I take the opportunity to thanks UGC for sanctioning this MRP.

Thanking You,

Yours Sincerely

Dr. Rajesh Singh Nirwan (PI)
Department of Botany
Seth Motilal (PG) College
Jhunjhunu-333001 (Raj)

Principal

Annexure-1a

UNIVERSITY GRANTS COMMISSION

CENTRAL REGIONAL OFFICE
TAWA COMPLEX (BITTAN MARKET)
E-5 ARERA COLONY, BHOPAL-16

REPORT OF THE WORK DONE UNDER THE SCHEME SUPPORT FOR MINOR RESEARCH PROJECT.

1. Name of the Principal Investigator: **Dr. Rajesh Singh Nirwan**
2. Number and date of sanction letter under which the project was sanctioned: **: MS-49/304044/XII/14-15/CRO Dated: 01May, 2015**
3. Title of research project: **Establishing optimum *in vitro* culture conditions for raising the morphogenic cultures with high regeneration potential in major millets.**
4. If working for the doctorate degree please state whether the thesis has been completed/ submitted. If not the approximate date by which it is likely to be submitted: **NA**
5. whether the paper related to the approved project have been published. If yes give details of paper and name of journal on which it has been accepted /published. Manuscript of paper entitled, **"Incorporation of heavy metals in basal nutrient media affect the efficiency of callus induction and plant regeneration in *Sorghum bicolor* (L.) Moench"** has **c**ommunicated to be published in journal of national repute.
6. If project has not been completed please indicate the approximate time by which it is likely to be completed and attach a summary of work done so far. : **Completed**
7. If project has been completed please enclose a consolidated report: **attached on separate sheet.**

SIGNATURE OF THE PI **PRINCIPAL**

Annexure-1b

UNIVERSITY GRANTS COMMISSION

CENTRAL REGIONAL OFFICE
TAWA COMPLEX (BITTAN MARKET)
E-5 ARERA COLONY, BHOPAL-16

PROJECT REPORT FOR MINOR RESEARCH PROJECTS

(To be furnished in duplicate by head of Department)

Progress report for the year: July 2015 to June 2017

1. Name of the Principal Investigator: **Dr. Rajesh Singh Nirwan**

2. Number and date of sanction letter under which the project was sanctioned:

MS-49/304044/XII/14-15 dated: 01 May, 2015

Non- recurring:

Items	Total grant allocated	Total grant received from UGC	Total expenditure incurred under the project	Utilization certificate furnished
Books and journals	30,000/-	30,000/-	29777/-	YES
Equipments	150000/-	150000/-	146200/-	

Recurring:

Items	Total grant allocated	Total grant received from UGC	Total expenditure incurred under the project	Utilization certificate furnished
Travel/field work	20000/-	10000/-	19800/-	YES
Contingency	50,000/-	25,000/-	49586/-	
Chemical /glassware	170000/-	85000/-	171171/-	
Special needs	10,000/-	5000/-	10,900/-	
Total	**4,30,000/-**	**3,05,000/-**	**4,27,434/-**	

Research paper published so far in referred journals during the project: **communicated**

National level conference/Seminar attended: 1

Certified that the program of research work done by Dr. Rajesh Singh Nirwan is satisfactory.

Place: **Signature Principal**

Annexure-IIa

UNIVERSITY GRANTS COMMISSION

CENTRAL REGIONAL OFFICE
TAWA COMPLEX (BITTAN MARKET)
E-5 ARERA COLONY, BHOPAL-16

STATEMENT OF EXPENDITURE IN RESPECT OF MINOR RESEARCH PROJECT UNDER THE SCHEME FOR RESEARCH IN HUMANITY/SOCIAL SCIENCE/ LANGUAGE /SCIENCE

1. Name of the Principal Investigator: **Dr. Rajesh Singh Nirwan**
2. Number and date of sanction letter under which the project was sanctioned: MS-49/304044/XII/14-15/ CRO Dated**: 01 may 2015**
3. Title of project: "**Establishing optimum *in vitro* culture conditions for raising the morphogenic cultures with high regeneration potential in major millets".**
4. Period of utilization from: 1 **July, 2015 to 30 June, 2017**
5. Details of expenditure:

Non recurring:

Items	Total grant allocated	Total grant received from UGC	Total expenditure incurred under the project
Books and journals	30,000/-	30,000/-	29777/-
Equipments	150000/-	150000/-	146200/-

Recurring:

Items	Total grant allocated	Total grant received from UGC	Total expenditure incurred under the project
Travel/field work	20000/-	10000/-	19800/-
Contingency	50,000/-	25,000/-	49586/-
Chemical /glassware	170000/-	85000/-	171171/-
Special needs	10,000/-	5000/-	10,900
Total	**4,30,000/-**	**3,05000/-**	**4,27,434/-**

If as a result of check or audit objection some regularizes is noticed at a later date action will be taken to refund, adjust or regularizes the objected amounts

SIGNATURE OF PI **PRINCIPAL**

Annexure-IIb

UNIVERSITY GRANTS COMMISSION

CENTRAL REGIONAL OFFICE
TAWA COMPLEX (BITTAN MARKET)
E-5 ARERA COLONY, BHOPAL-16

(UTILIZATION CERTIFICATE IN RESPECT OF ITEMS/PROGRAMS WHICH ARE IN PROGRESS/ COMPLETED)

It is certified that the university Grants Commission sanctioned Rs 4,30,000/-Vide letter no.MS-49/304044/14-15/CRO dated: 01 May, 2015 for minor research project which under implementation has been completed. It is certified that the progress of expenditure on the scheme is as under:

A. Non recurring:

Items	**Cost approved by UGC**	**Grant released from UGC so far**	**UGC sanction letter and date**	**Actual expenditure as on date**	**Remark**
Books and journals	30,000/-	30,000/-	MS-49/304044/XII /14-15/CRO/Date: 01may,2015	29777/-	
Equipments	150000/-	150000/-		146200/-	

B. Recurring:

Items	**Cost approved by UGC**	**Grant released from UGC so far**	**UGC sanction letter and date**	**Actual expenditure as on date**	**Remark**
Travel/field work	20000/-	10000/-	MS-49/304044/XII /14-15/CRO/Date: 01may,2015	19800/-	
Contingency	50,000/-	25000/-		49586/-	
Chemical /glassware	170000/-	85000/-		171171/-	
Special needs	10,000/-	5000/-		10,900/-	
Total	**4,30,000/-**	**3,05000/-**		**427434/-**	

C. This certificate is based on audited/unaudited statement of expenditure: **Audited**

D. The items of stock have been taken in the asset (ledger/register) of institution.

SIGNATURE PI **PRINCIPAL**

Annexure-IV

UNIVERSITY GRANTS COMMISSION

CENTRAL REGIONAL OFFICE
TAWA COMPLEX (BITTAN MARKET)
E-5 ARERA COLONY, BHOPAL-16

UTILIZATION CERTIFICATE

Certified that the sanctioned grant of Rs.4,30,000/- received from the University grants Commission under the scheme of support for minor research project in the science entitled, "**Establishing optimum *in vitro* culture conditions for raising the morphogenic cultures with high regeneration potential in major millets.**" vide University grants Commission letter no. MS-49/304044/XII/14-15/CRO dated: 01 May, 2015, out of which Rs.4,27,434/- has been fully utilized for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the University grants Commission.

SIGNATURE OF PI **STATUTORY AUDITOR/CA** **PRINCIPAL**

TITLE OF MINOR RESEARCH PROJECT: ESTABLISHIN OPTIMUM IN VITRO CULTURE CONDITIONS FOR RAISING MORPHOGENIC CULTURES WITH HIGH REGENERATION POTENTIAL IN MAJOR MILLETS.

SUMMARY:

The major millets or coarse cereals including the *Sorghum bicolor* and *Pennisetum glaucum* are crops of extreme genetic diversity. The major millets are endowed with several important features that make them ideal for basic and applied research. Realizing the importance of these crop plants and to explore the inherent untapped potential the present study was undertaken with the objective to initiate and establish the morphogenic cultures with high regeneration capability. The different varieties of both the sorghum and pennisetum were screened for their in vitro response. Optimization of nutrient medium affects the morphogenic potential and regeneration capacity of cultures. Different nutrient media like MS medium (1962), B5 medium (1968), N6 medium (1975) were screened for in vitro response. The MS medium was found suitable and it supported the in vitro growth of explants. Maximum rhizogenesis was observed in cultures with N6 medium. Different explants viz. mature seeds, mature and immature embryos, leaf bases of both sorghum and pennisetum were used to analyses their callusing and regeneration behavior under in vitro conditions. The explants were inoculated on MS medium fortified with different auxins alone viz.2,4-D. 2,4,5-T, pCPA, pic, IAA, IBA and NAA (0.5-5.0mg/l) and their combinations with cytokinin BAP/Kn (0.5- 2.0mg/). Among the broad category of explants immature embryo was found best responsive explants on MS medium supplemented with moderate amount of auxins 2,4-D, pCPA (2.0mg/l) and lower concentrations of cytokinin BAP/Kn (0.5mg/l) in terms of fresh weight of callus and number of regenerants per callus culture in sorghum while in case of pennisetum the best concentration of plant growth regulators were 2,4-D (3.0mg/l) and BAP (0.5mg/l). The best regeneration response was observed for the callus induced on pCPA (3.0mg/l) and Kn /BAP (0.5mg/l) supplemented medium. The green, compact nodular callus was tested for its regeneration potential by culturing it on plain MS medium and MS medium containing different plant growth regulators. The best regeneration response was observed for sorghum on MS medium supplemented with Kn (2.0mg/l) plus IAA (0.5mg/l) while in pennisetum it was on MS medium fortified with Kn (0.5mg/l) plus IAA (1.0mg/l). The degree of in vitro response could be increased by exogenous supply of some growth adjuvants like glutamine (150 mg/l), silver nitrate 2.0, PVP 150mg/l in sorghum while in pennisetum it was glutamine (100mg/l) and silver nitrate (3.0mg/l), PVP (100mg/l). Exogenous supplementation of

medium with activated charcoal (20 mg/l) in sorghum and (30mg/l) in pennisetum improved the amount of fresh weight of callus and texture of calli. Addition of 3 % (w/v) sucrose in the nutrient medium as carbon source served best to support the in vitro growth for sorghum and pennisetum explants derived cultures.

Effect of different heavy metals like copper, Zinc, Iron, Molybdenum, cobalt, nickel and silver was also analyzed for sorghum in vitro cultures. Incorporation of higher levels of copper (4X-10X) than the standard level in MS medium (0.025mg/l) supported maximum growth of cultures and improved the regeneration potential of cultures. Normal levels of Zinc (8.6mg/l) supported the maximum proliferation of callus but regeneration potential was higher for the callus induced on higher amount of Zinc. Standard level of iron- EDTA was best suited for callus induction and plant regeneration. The amount of callus was higher on medium devoid of Molybdenum but it was rhizogenic in nature. Healthy shoots were cultured to half strength MS medium and full strength MS medium with some alterations of sucrose and addition of auxin (IAA) in rooting medium to develop efficient root system. Best rooting response was observed for pennisetum on half MS medium plus sucrose 3% (w/v) and in sorghum it was half MS medium plus IAA (0.5mg/l). These combinations favored the development of profuse root system.

CONTRIBUTION TO THE SOCIETY:

The major millets including the Sorghum and Pennisetum are staple food crop in the poorer and dry regions of world. They are of great local importance as staples and as reserve crops in marginal areas. High frequency regeneration of plants from cells and tissue is an essential component of biotechnology that is required for genetic manipulation and novel source of variability with a great potential for crop improvement. Availability of high regeneration protocol is a prerequisite for application of genetic engineering. The protocol developed may serve as a tool in improvement of these plants. The optimization of in vitro conditions by selection of proper media and explants will play an important role in establishing the highly regenerable cultures in the plants which are generally recalcitrant. The genetic improvement of existing genotype of crop plants may serve in better way to provide quality food and fodder to dry land area people. Besides this, the proposed work has also given an understanding to the students about the whole process of plant tissue culture techniques. The students could apply their experience on conservation and micro propagation of local endangered plants.

Detail Project Report

TITLE OF MINOR RESEARCH PROJECT: ESTABLISHING OPTIMUM *IN VITRO* CULTURE CONDITIONS FOR RAISING THE MORPHOGENIC CULTURES WITH HIGH REGENERATION POTENTIAL IN MAJOR MILLETS.

INTRODUCTION:

Millets stand next to cereals regarding their consumption as source of food stuff for a large population all over the world. Millet is a group of small grained cereal crop known as coarse grains. These crops have been divided in two groups major and minor millets. Major millets (Coarse cereals) include jowar (*Sorghum bicolor*) and bajra (*Pennisetum glaucum*) other important cereals include ragi, kodomillet, foxtail millet, barnyard millet and little millet. They posses characteristic ability to survive and tolerate the adverse environmental conditions especially continuous drought and alkalinity. Millets have been utilized for human consumption since old age. Millets are staple food crop in the poorer and dry regions of world. They are well suited to areas with inadequate moisture and poor soil fertility (Baker, 2003). They are of great local importance as staples and as reserve crops in marginal areas. Millets are nutritionally comparable and even superior to major cereals in terms of energy value, proteins, fat and minerals. They are also good source of phytochemicals and phenolic acids (Qureshi *et al., 2000*). Millets have considerable potential for use as an ingredient in food and beverages. As they are gluten free and suitable for celiac. The traditional food and snack food have been produced from sorghum and minor millets. They are lower in proteins but richer in fat than wheat. These are comparable with rice in nutritional value. China, India and Africa are the main millet producing countries in world.

Pearl millet (*Pennisetum glaucum*) is the sixth most important crop of all the world's cereals. Descended from a wild West African grass, it was domesticated more than 4,000 years ago,

probably in what is now the heart of the Sahara. Long ago it spread from its homeland to East Africa and thence to India. Both places adopted it eagerly and it became a staple.

Today, pearl millet is so important that it is planted on some 14 million hectares in Africa and 14 million hectares in Asia. Global production of its grain probably exceeds 10 million tons a year, to which India contributes nearly half. At least 500 million people depend on pearl millet for their lives.

Despite its importance, however, pearl millet can be considered a "lost" crop because its untapped potential is still vast. It lags behind sorghum and far behind the other major grains in its genetic development. It is almost entirely a subsistence crop. Most taxonomists today believe that the most valid name for cultivated pearl millet is Pennisetum glaucum (L.) R.Br. Common synonyms are *Pennisetum typhoides* and *Pennisetum americanum.* The crop is also known as "bulrush millet" and in India it is normally called "Bajra." Today it is India's fourth most important cereal, surpassed only by rice, wheat, and sorghum. *Bajra* is currently grown on almost 10 percent of India's food-grain area, and it yields about 5 percent of the country's cereal food. Rajasthan, Maharashtra, Gujarat, and Uttar Pradesh and Haryana account for nearly 80 percent of the 14 million hectares planted and 70 percent of the 5 million tons of pearl millet grain produced each year. In India it is fourth most important food crop after rice, wheat and sorghum.

Pearl millet is supremely adapted to heat and aridity and, for its entire current decline, seems likely to spring back as the world gets hotter and drier. Perhaps the best of all "life-support" grains, pearl millet thrives where habitats are harsh. Of all the major cereals, it is the one most able to tolerate extremes of heat and drought. It yields reliably in regions too hot and too dry to consistently support good yields of maize or even sorghum.

Indians commonly grind pearl millet and make the flour into cakes or unleavened bread (*chapati*). Some goes into porridges, which may be thin or thick. Much is cooked like rice. Small quantities of the grains are used for feeding cattle and poultry, the plant is more often fed to animals as a green fodder. It is well suited for this purpose because it is quick-growing, tillers very freely, lends itself to multiple cutting, and usually has thin and succulent stems. The pearl millet grain is nutritious. It has no husk, no tannin, contains 5-7 percent oil, and has higher protein and energy levels than maize or sorghum. In general, pearl millet has higher protein content than other cereals grown under similar conditions. Millets do not contain gluten (Leder, 2004). They have shown antiproliferative property and might have potential in prevention of cancer (Chandrasekara and Shahidi, 2011).

Sorghum is fifth leading cereal crop after wheat rice maize and barley. It is the crop with extreme genetic diversity but an overriding characteristic is its tolerance to heat and drought. As a result of tolerance sorghum has emerged as the food grain most widely utilized for human consumption in dry regions of world India.

So it is now necessary to reestablish the importance of pearl millet and sorghum in the drier areas and to do so we must make the production of these crops attractive enough so that they can compete with maize, not only in the worst and most severe droughts but in at least a majority of years.

The major millets are endowed with several features that make them ideal for basic and applied research. These include the low chromosome number with large size, short life cycle, easy selfing and crossing, plasticity for tillering, large number of seeds per panicle and hard nature of crop which can withstand stress environment.

Being the crops of semiarid tropics the major millets suffers from a number of constraints because of which their area and production has been fluctuating year to year. The major constraints are drought, low fertility and yield reducing biotic factors. It is imperative to make these crops remunerative under prevalent constraints through biotechnology.

Genetic variations are very important for crop improvement. Plant cell and tissue culture provides increased variability. This genetic variability is expressed in form of variant traits in regenerated plants. The effective use of tissue culture technique for *in vitro* selection and possibility of genetic introgression depends upon the ability to initiate and establish callus cultures capable of plant regeneration.

Regeneration of plants from cells and tissue is an essential component of biotechnology that is required for genetic manipulation and improvement of crop plants. The tissue culture technology may provide rich and novel source of variability with a great potential for crop improvement. A high rate of regeneration from callus cultures is prerequisite for the use of tissue culture as a tool in crop improvement. Though the cereals and millets have been considered as recalcitrant to plant tissue culture but selection of proper media and explants play an important role in establishing the regenerable tissue cultures. Growth and pattern of morphogenesis of plant tissues under *in vitro* conditions are governed by composition of nutrient media. The composition of nutrient medium plays an important role in establishing healthy tissue cultures. It has been shown that nutrient levels in the medium have profound effect on callus induction and plant regeneration. During the course of *in vitro* growth and differentiation interplay occurs between nutrients and plant growth regulators. Appropriate levels of nutrients in medium may partially substitute the requirement of plant growth regulators. Several reviews have been published on extensive progress achieved in plant tissue cultures in cereals and millets

(Bhaskaran and smith 1990; Vasil and Vasil 1992; Kothari and Chandra 1995; Hansen and Wright 1997; Rapellin *et al*., 2001; Jankiraman *et al*., 2002, Sonia plaza and Zrerihun Tadele 2012). Realizing the importance of major millets in dry land regions and inherent immense scope of improvement of these crops the present study was undertaken with following objectives for optimization of *in vitro* culture conditions and levels of inorganic micro-nutrient so as to raise highly morphogenic cultures and increase the regeneration potential of these crops.

OBJECTIVES:

(1) Screening of major millet varieties best suited for desert conditions and in vitro response.

(2) Establishment and maintenance of embryogenic cultures.

(3) Optimization of in vitro conditions:

   a. Selection of best explants among a broad category of explants.
   b. Development of a technique of sterilization.
   c. Screening of nutrient media and optimization of nutrient concentrations.
   d. Selection of ideal combinations of plant growth regulators and their appropriate concentrations.

(4) Replacement of requirement of exogenous supply of Plant growth regulators in to medium by media constituents.

(5) Evaluation of in vitro regeneration response.

MATERIAL AND METHODS:

BOTANICAL DESCRIPTION OF PLANTS:

Pearl millet is an annual plant of family poaceae with fine and long stem Pearl millet is an upright bunch grass that tillers from the base and has an extensive root system that provides drought tolerance. Stems are 1/2–1 inch diameter. It is a leafy plant with leaf blades that are 8–40 inches long and 1/2–3 inches wide. The ligule, or junction of leaf blade to leaf sheath, is a fringe of hairs 0.08–0.1 inch long. The sheath has very sparse hairs at the base of the collar and is often hairless. The inflorescence (flower) is a single raceme 4–20 inches long. The fruit (or caryopsis) is cylindrical, white or pearl in color, or sometimes yellow or brown, and occasionally purple.

Sorghum is a coarse, erect grass. Its growth characteristics can vary quite drastically and a fully matured plant can range in height from 0.45 to over 5 m. Life cycle: annual. Growth habit: 4-8 feet tall; resembles corn, but smaller Leaves: 1-2.5 inch wide blades with white midveins Inflorescence: July - October. Large panicles of rounded, shiny black or red seeds that shatter easily. Stem: smooth. Root: fibrous root system. Leaves broad and coarse, similar in shape to those of corn but shorter and wider; blades glabrous and waxy; sheaths encircle Culm and have overlapping margins; panicle erect, sometimes recurved, usually compact in most grain sorghums and more open in forage types; seed covered by glumes that may or may not be removed by threshing; prop roots may grow from culm nodes; bud at each node from which a tiller may grow; seeds white, yellow, red, or brown; panicle with spikelets.

EXPLANT SELECTION:

Seeds of major millets (Sorghum and pearl millet) were utilized for morphogenetic studies. Detailed study was carried out using different explants viz. seeds, mature and immature embryos, leaves, nodal segments, shoot apices etc.

Mature seeds obtained from field were pre sterilized and soaked in sterile distilled water under aseptic conditions for 40 hrs to obtain immature embryo explants from these seeds. Immature seeds were collected from the field grown plants. Cotyledons were dissected out after sterilization under aseptic conditions.

Seedling explants were obtained from the in vitro germinated seeds after 5 days of their germination. Leaves were taken out of the seedling and small pieces of leaf bases were cut and cultured on nutrient medium.

STERILIZATION OF PLANT MATERIAL

Seeds were taken in beaker and washed in running tap water after washing, seeds were treated with 70% (v/v) ethyl alcohol for 1 min further sterilization was carried out in laminar air flow cabinet by immersing the seeds in 0.1% (v/v) aqueous solution of $HgCl_2$ for 3 min and they were washed 3 times with double distilled water after sterilization the seeds were cultured on to the medium. Mature and immature explants were obtained by dissecting the seeds aseptically.

CULTURE MEDIUM

In the present study different types of nutrient media (MS Medium, N6 medium and B_5 Medium) were screened for their best suitability to the cultures. MS medium (Murashige and Skoog, 1962) was evaluated for the maximum amount of morphogenic cultures. Stock solutions of various macronutrients, micronutrient and vitamins at various concentrations 50X, 100X ere prepared by dissolving appropriate weighed amount of substance in measured volume of distilled water. Stock solutions were stored in refrigerator. Stock solutions were used within one month of the preparation. Each stock solution for a group of substances was labeled with alphabetical letters. Stock solutions of growth regulators and other chemicals were prepared by dissolving auxins

(i.e. 2, 4-D, 2,4,5-T, pCPA, PIC, NAA, IBA, IAA, etc.) in few drops of ethyl alcohol and cytokinins (BAP or Kn) in 2-3 drops of 1N HCl, Subsequently final volume was made up by adding distilled water, stock solutions of PGRs were also prepared by dissolving in a few drops of alcohol before adding required distilled water to make final volume (20mg PGR was dissolved in 100 ml distilled water).

Nutrient Medium was prepared by adding the appropriate volume of various stock solutions. Required amount of sucrose (30gm/L) was dissolved separately in small quantity of distilled water and then added to mixture of stock solutions. Final volume was made up by adding DW. Growth adjuvants were added from already prepared stock solutions in different concentrations. Nutrient Medium was dispensed to glass beaker according to the quantity of medium required. Agar 0.8-1% (w/v) Qualigens, Hi media, or BDH or phytagel 0.2% (w/v) sigma was added to medium. Finally the pH of the medium was adjusted to 5.8 by adding 1 N HCl or 1N NaOH. Medium was heated to melt agar or phytagel and then the medium will be dispensed into culture vessels (test tube or conical flask) 10-20 ml medium was dispensed in to test tube and 40-48 ml medium was dispensed into Borosil Erlenmeyer flask (100 ml). Flasks or test tubes were plugged tightly with non absorbent cotton and mouth wrapped by paper. Nutrient Medium was autoclaved at 121^0C temp. and 1.2-1 3 kg/cm^2 pressures for 15 minutes.

ASEPTIC CONDITIONS

Aseptic inoculations of explants on nutrient media were carried out inside the laminar airflow cabinet. Cabinet was irradiated by UV rays from germicidal lamp (15W, Sankyo Denki Co. Japan) for 45-60 minutes before starting the inoculation work. Before switching on the UV light

the slab of cabinet was swabbed properly with rectified spirit. All the required surgical such as scalpel needle, forceps etc. were kept in spirit column.

INCUBATION

After inoculation the flasks and test tubes were incubated in the culture chamber. Temperature of chamber was maintained up to 26 ± 2^0C using air conditioner and photo thermal controllers. Light of 1600 LUX was provided from fluorescent tube of 40W and incandescent bulbs 40W. A photoperiod of 16 hrs alternating with 8 hr dark period was regulated with the help of timer

EQUIPMENT AND APPARATUS

Morphogenetic studies were carried out by setting experiments using following equipment and apparatus

Glassware

a. Conical flasks –Erlenmeyer (100 ml -1000 ml)Round Bottom flasks (250 ml -1000 ml)Test Tubes (20x150 mm; 250150 mm and 38x200 mm)Beakers (100-2000 ml)Measuring cylinder (10-1000 ml)Pipettes (2ul-10 ml)Petri dishes (100x50 and 150 x75 ml)Reagent bottles (100-500 ml, both transparent and amber colored)

1. Water distillation unit Local –fabricated 2Hot plates (Elite, India)

3. Heater (Dollar, India) 4 Electronic balance (Adair –Dutt (0.0001g-180g) (Adair –Dutt (0.001g-220g)

5.Crude balance (0.01-100g)

6. Autoclave (SEW, India)

7. Laminar air flow cabinet (Thermadyne, India)

8. Electronic platform shakers (Infors with control devices, ORBITEK, India)

9. Incubator shaker (Adolfkuhner-Switzerland, ORBITEK, India)

10. Oven (SEW, India)

11. Centrifuge (Sigma USA, REMI, India)

12. Stereomicroscope (Nikon, Japan)

13. Compound microscope (Nikon, Japan)

14. Monocular microscope (Laborned, India)

15. pH Mater (Control Dynamics, India)

16. Refrigerator (Godrej, Kelvinator and Whirlpool, India)

17. Microtome (Weswox, India)

18. Photography equipment (Nikon Stereomicroscope and slide microscope 19 photographic attachments)

20 Other accessories

Forceps (Different sizes) Kelker, India, Scalpels, mounted and metallic needles, scissors, spatula, spirit lamp, aluminium foil, parafilm, sterile

disposable petriplates (35-60 mm diameter Tarson, Hi Media India, Plastic Measuring cylinders (Cap 100 ml -1000 ml) plastic beakers.

CALLUS INDUCTION AND PLANT REGENERATION

Different explants were inoculated for raising primary cultures on MS medium supplemented with different levels of plant growth regulators and the growth adjuvant Visual observations were made up to 4 to 6 weeks. Fresh weight was taken by weighing the tissue after 6 weeks.

Plant regeneration was achieved by transferring the morphogenic callus on regeneration medium. Observations were made by counting the number of shoots and their length when shoots had elongated properly after 4 weeks.

SUBCULTURING PROCESS

Primary morphogenic callus was maintained by continuous short term sub culturing process. Callus with non-embryogenic, watery appearance was discarded and hard, nodular callus with green patches was maintained on fresh medium supplemented with 2,4-D (2.0mg/1) plus kinetin (0.05 mg/1) or BAP (.05 mg/1) . Certain other growth adjuvants were also tried to evaluate the effect of amino acids in maintenance of morphogenic callus at 3 weeks interval. Data were recorded after every 3 weeks.

ROOTING PROCESS

Regenerated shoots were transferred to half strength MS medium or MS medium and other modifications of MS medium supplemented with IAA and different concentration of sucrose. Medium was solidified with phytagel. Observations were made after 4weeks, by counting the number and length of roots per shoot.

FIELD TRANSFER OF REGENERATED PLANTLETS

Regenerated plantlets with efficient root system were taken out from the culture tubes. Solidified medium was removed under running tap water. Plantlets were directly transferred into earthen pots containing soil and compost in the ratio of 1:1, pre-acclimatization was not required for field transfer.

OBSERVATIONS AND RESULTS:

Screening of cultivars for in vitro response:

Mature seeds of both sorghum and pennisetum including the inbreed and hybrid varieties were cultured on to MS medium fortified with different concentration of 2,4-D (0.5-5.0 mg/l) and Kn (0.5-2.0mg/l). After 3 to 5 days the inoculated seeds swelled and germinated. The germinating seeds start forming callus. The callus was of two type; non embryogenic with watery, soft and transluscent cells and embryogenic type having green nodular with shiny surface cells. The embryogenic callus was transferred to regeneration medium supplemented with various concentrations of cytokinins. Best in vitro response in terms of fresh weight of callus and plants regenerated was observed for variety of sorghum SPV245 and variety Raj171 for pennisetum. These varieties were further used to investigate the morphogenic response and to optimize the in vitro culture condition for raising the morphogenic cultures.

Explant selection:

Selection of a suitable explant among the broad category of explants is very important to optimize the in vitro response. Different explants of selected varieties like seeds, mature and immature embryos, leaf bases etc were cultured on MS medium containing different auxins (2,4-D, 2,4,5-T, pCPA, Pic, IAA, IBA and NAA) alone or in combination with cytokinins (BAP/Kn) and with other growth adjuvants. The primary callus induced from different explants was cultured on regeneration medium to examine the regeneration potential along with every subculture**.**

A) **Seed culture:** Surface sterilized seeds of selected varieties were cultured on MS medium supplemented with auxins alone and their combination with cytokinins. Callusing response was observed on MS medium supplemented with 2,4-D, 2,4,5-T, pCPA, Pic (0.5- 5.0 mg/l). Seeds start germination after 3 to 5 days of inoculation after 6 days callus

formation took place. The amount of callus in terms of fresh weight varied according to type and concentration of plant growth regulator. Seed response was best for Sorghum at 2,4-D and pCPA (2.0mg/l) and 2,4,5-T (0.5mg/l) and for pennisetum it was at 2,4-D (2.0mg/l) pCPA (3.0mg/l) and 2,4,5-T (3.0mg/l). Maximum amount of callus was watery and produced roots incultures. The remarkable decrease was observed with higher concentrations of auxins in nutrient medium. Some auxins like IAA, IBA and NAA induced germination of seeds. Cytokinin alone supplemented medium supported only germination of seeds and no callus formation was observed both in sorghum and pennisetum. Addition of BAP could not raise embryogenecity in cultures. The callus was watery and soft and dirty in appearance. The best callusing response was observed on medium supplemented with 2,4-D and pCPA (2.0mg/l) and kn/BAP (0.5mg/l). Higher concentration of auxins and cytokonins were found inhibitory both in Sorghum and Pennisetum.

B) **Mature embryos:** Mature embryos excised from presoaked seeds of both sorghum and pennisetum were cultured on MS medium supplemented with different concentrations of auxins and cytokinin either alone and in combinations. In all cultured embryos swelling took place after 3 days of culture. Callusing started from the germinating part which comes in contact to medium. This response was observed in all cultures containing 2,4-D, 2,45-T, pCPA (0.5-5.0mg/l). The callus seems to be embryogenic but later turned in to rhizogenic. Callus induced on to media fortified with 2,4-D (2.0mg/l) was best both for sorghum and pennisetum in terms of fresh weight. Addition of Pic (3.0mg/l) to medium could increase the amount of callus. Every concentration of 2,4,5-T supported callus formation but it was non-embryogenic. Addition of pCPA (2.0mg/l) was found with

positive effects while incorporation of IAA, IBA exhibited the germination of embryos. All concentrations of NAA (0.5-10mg/l) induced multiple shoot formation without callusing response.

Addition of cytokinins (Kn/BAP) in to callus induction medium resulted in germination of embryos. Mature embryos cultured on to MS medium supplemented with 2,4,-D, pCPA (0.5-5.0mg/l) in combination with cytokinin (BAP/Kn; 0.5-2.0mg/l) formed morphogenic callus with green, compact, nodular structures within callus. The callus also had small proportion of non embryogenic cells with watery, soft and transluscent texture.

Best callusing response was found with medium containing 2,4-D and pCPA (2.0mg/l) and Kn/BAP (0.5mg/l) in terms of fresh weight of callus. Higher concentrations of auxins caused rhizogenesis and higher concentrations of cytokinins induced shoot formation in cultures. Multiple shoot formation was observed on NAA supplemented medium in sorghum. Increase in concentration of NAA increased the number of shoots. Maximum shoots were produced at NAA 10.0mg/l.

C) **Immature embryo culture:** Immature embryos of both Sorghum and Pennisetum were excised from young caryopses when they were at their milky stage. They were cultured on MS medium supplemented with different auxins viz. 2,4-D, 2,4,5-T, pCPA, Pic, IAA,IBA and NAA (0.5-5.0 mg/l). Addition of 2,4-D and pCPA (2.0 mg/l) induced maximum callusing response. Lower and higher concentrations could not increase the in vitro callusing response. Higher concentrations were found inhibitory for callusing. The amount of callus was scanty for initial 3 day but it later increased due to luxuriant proliferation of callus. Higher concentrations of auxins induced release of phenolic compounds in medium especially in case of sorghum. Leaching of phenolic compounds

caused the browning of medium. This exhibited detrimental effect on growth of callus. Immature embryos were also cultured on MS medium fortified with auxins in combination with cytokinin. A combination of auxins (2,4-D or pCPA; 2.0mg/l) with BAP was highly responsive where good amount of morphogenic callus was formed. Moderate amount of callus was formed on other auxin supplemented medium. Lower concentrations of BAP/Kn (0.5mg/l) favored the production of higher amount of callus.

D) **Leaf bases:** Leaf bases were excised from 5-7 days old seedlings grown under in vitro conditions. They were cultured on to MS medium fortified with different auxins viz. 2,4-D, 2,4,5-T, pCPA, Pic, IAA,IBA and NAA (0.5-5.0 mg/l). Leaf bases which were taken from young age seedling (5days) were found better for callus induction. Leaf bases were initially swelled curled and starts callusing. The callus formed on auxin supplemented medium was watery, translucent and non-morphogenic. Leaf bases cultured on toMS medium supplemented with 2,4-D (0.5-5.0mg/l) and BAP/Kn (0.5 mg/l) produced only small amount of callus. The cultures start leaching of phenolic compounds and cells showed higher degree of necrotization. Supplementation of medium with IAA,IBA and NAA did not favored callusing response.

Regeneration response of primary callus derived from different explants:

Different explants derived callus was cultured onto plain MS medium and the medium supplemented with different plant growth regulators at their varied concentrations. The primary callus induced on callus induction medium with different hormonal manipulations had both morphogenic and nonmorphogenic cells. The only morphogenic callus was cultured onto MS medium with different cytokinins (BAP/Kn; 0.2-2.0mg/l) to assess the morphogenic potential of callus and devise an efficient regeneration medium.

The non-embryogenic watery, transluscent callus was discarded. Though the addition of both cytokinins to medium supported emergence and proliferation of shoots. The regenerants number was higher on the medium containing the BAP (2.0mg/l) plus IAA (0.5mg/l) but shoots were weak. The shoots produced on MS medium supplemented with Kn (2.0mg/l) plus IAA (0.5 mg/l) were stout and healthy in sorghum. In Pennisetum the maximum number of regenerants was observed on IAA (1.0 mg/l) plus Kn (0.5 mg/l). The percentage of regeneration efficiency was tried to enhance with addition of antioxidants like PVP, silver nitrate and aspartic acid. Incorporation of PVP 150mg/l could a bit prevent the leaching of phenolic compound and supported the healthy growth of shoots. These combinations of hormones were further used as regeneration medium.

Effect of different growth adjutants on callus induction, maintenance and plant regeneration:

Effects of various growth adjutants like ascorbic acid, yeast extract, activated charcoal, glutamine, polyvinyl pyrolidone (50- 150mg/l) silver nitrate (1-5 mg/l) was evaluated both in callus induction and plant regeneration. The growth adjuvant were added to callus induction medium containing 2,4-D (2.0 mg/l) and Kn (0.5 mg/l). The incorporation of PVP (150mg/l) glutamine (100 mg/l) improved the growth of callus and number of shoots increased. Addition of PVP (150 mg/l) and silver nitrate (3.0mg/l) could reduce the browning of medium.

The primary morphogenic callus induced by mature embryos could be maintained by continuous subculturing at 3 week intervals on lower concentrations of auxins and cytokinin with added amount of PVP (100mg/l) and glutamine (100mg/l) in case of

sorghum. In case of pennisetum embryogenic callus proliferated well on lower concentration of auxins alone.

Effect of Heavy Metals on Callus Induction and Plant Regeneration:

Experiments were conducted to study the effects of heavy metals viz, $CuSo_4$, $ZnSO_4$. $MnSo_4$, Fe-EDTA, Na_2MoO_4, $CoCl_2$, $NiCl_2$ and $AgNO_3$ on in vitro morphogenesis in Sorghum bicolor. Mature embryos were cultured on MS medium supplemented with 2,4-D (2.0 mg/l) + Kn (0.5 mg/l) and various concentrations of heavy metals in separated cultures. Mature embryos swelled and callusing started within 4-5 days. After 4-6 weeks of mature embryo inoculation, good amount of callus was formed. Two diference morphotypes of callus were observed. It was hard, compact, nodular morphogenic with the patches of soft, watery, translucent non-embryogenic type of callus. After studying the effect of heavy metals on callus incudction and making observations, primary embryogenic callus was transferred to regeneration medium containing the normal leaves of particular microelement and on medium with modified leaves of microelements. Thus, varied levels of microelements were used both for callus induction and plant regeneration.

Copper Sulphate ($CuSO_4$):

Copper is an essential microelement, which takes part in several biochemical reactions of plant metabolism. It is present in very small amount (0.025mg/l) in MS medium. Levels of $CuSO_4$ were raised up to 50x to the normal level of MS medium both for callus induction and plant regeneration. The various levels of $CuSO_4$ in callus induction and plant regeneration medium were (0, 0.025-2.5 mg/l)

Amount of embryogenic callus induced on media supplemented with higher levels of copper was remarkable. It was maximum of copper sulphate (2.5mg/l). Embryogenic callus induced on each treatment was transferred to normal and on modified regeneration medium enhanced plant regeneration was observed for increased levels of copper sulphate. Maximum number of shoots was reported on media supplemented with copper (10X) for the callus induced on same concentration of copper in callus induction medium. Regeneration response was poor on media devoid of copper sulphate..

Zinc Sulphate ($ZnSO_4$):

Mature embryos of sorghum were cultured onto MS medium with varied levels of $ZnSO_4$ (0, 8.6-34.4mg/l). Normal concentration of $ZnSO_4$, in MS medium is 8.6mg/l. after 6 weeks of culture morphogenic callus was transferred to regeneration media with normal and altered concentration of $ZnSO_4$. Although, normal level of $ZnSO_4$ (8.6mg/l) was found suitable for production of maximum amount of morphogenic callus, but number of plants regenerated was low. Absence of $ZnSO_4$ both from callus induction and plant regeneration medium proved inhibitory. Maximum number of regenerants was obtained from the callus induced on media supplemented with $ZnSO_4$ (8.6mg/l) was found suitable for production of maximum amount of morphogenic callus, but number of plants regenerated was low. Absence of $ZnSO_4$ both from callus induction and plant regeneration medium proved inhibitory. Maximum number of regenerants was obtained from the callus induced on media supplemented with $ZnSO_4$ (8.6mg/l). Thus, $ZnSO_4$ (8.6mg/l) was found optimum concentration for callus induction as well as plant regeneration. However, $ZnSO_4$ (34.4mg/l) was quite tolerable for callus induction and plant regeneration

Manganese Sulphate ($MnSo_4$):

Normal concentration of $MnSO_4$ in MS medium is (16.9mg/l). Callus induced on every concentration of $MnSO_4$ supplemented medium was soft, translucent with patches of green compact, nodular morphogenic callus. Morphologicial appearance of callus was almost similar in all treatments of $MnSO_4$. Less number of shoots developed with the callus induced on medium devoid of MnSO4 when transferred to regeneration medium containing normal level of $MnSO_4$. Increase in the level of $MnSO_4$ (4x) both in callus induction and plant regeneration media improved the formation of callus induction and plant regeneration media improve the formation of callus and production of regenerated per callus culture. However, shoots regenerated on higher levels of $MnSO_4$ were thin and weak.

Cobalt chloride ($CoCl_2$):

Being a microelement $CoCl_2$ in micro quantity i.e., 0.11□M(0.025mg/l) in MS medium. Its concentration was varied upto 50x is present in MS medium. Callus induced on each treatment of $CoCl_2$ was similar in morphological nature. Deficiency of $CoCl_2$ in callus induction medium decreased the amount of callus and no regeneration was achieved from this callus. Increase in concentration of $CoCl_2$ both in callus induction and plant regeneration medium had no striking effects for improvement in callus formation and in regenerability. Increase in concentration of $CoCl2_2$ resulted in formation of rhizogenic callus.

Sodium molibnate Na_2MoO_4:

Levels of Na_2MoO_4 used in callus induction and plant regeneration medium were (0, 0.25-1.0mg/l). Normal level of Na_2MoO_4 in MS medium is (0.25mg/l). After 3 days

of mature embryo inoculation, swelling started followed by callusing. Callus induced on all treatments was both morphogenic and non-embryogenic type. Maximum amount of callus in terms of fresh weight of callus was formed on media deficient of Na_2MoO_4 but regeneration response was not good. Callus formed was almost similar in fresh weight upto 4x of Na_2MoO_4. Higher-level decreased the fresh weight of callus. Regeneration efficiency was best for the callus induced on Na_2MoO_4 (0.5mg/l) and on modified levels of Na_2MoO_4 in regeneration medium. Increased levels of Na_2MoO_4 led to rhizogenesis. Very high level of Na_2MoO_4 in regeneration medium induced albino shoot formation. However, Na_2MoO_4 was tolerable upto 4x both for callus induction and plant regeneration.

Iron –EDTA (Fe-EDTA)

Different concentrations of Fe-EDTA used were (0, 27.8-111.2 mg/l) Normal level of Fe-EDTA in MS medium is 27.8mg/l. Normal level of Fe-EDTA (27.8mg/l) was found to be suitable both for callus induction and plant regeneration. The medium devoid of Fe-EDTA could not induce callusing. Addition and deletion of Fe-EDTA to the medium was found inhibitory for in vitro morphogenesis.

Nickel chloride ($NiCl_2$):

Varied levels of $NiCl_2$ were used (0, 0.10, 0.05, 2.1, 4.2, 8.4 µM). After 6 weeks of mature embryo inoculation, efficient amount of morphogenic callus was formed on $NiCl_2$ supplemented medium. NiCl2 (4.2 µM) was found more suitable for morphogenic callus induction response in terms of fresh weight of callus. Exogenous supplementation of $NiCl_2$ to regeneration medium improved the regenerability of callus cultures. Callus induced from all treatments was transferred to normal as well observed in the callus

induced on MS medium supplemented with $NiCl_2$ (4.2 µM). Addition of $NiCl_2$ (4.2µ M), induced albino shoot formation. Poor regeneration response was observed when callus induced on $NiCl_2$ supplemented media was transferred to normal regeneration media

Silver nitrate ($AgNO_3$):

Mature embryos were cultured onto MS medium with exogenously supplemented AgNo3. Various levels of $AgNo_3$ were used in present study. These are 0, 2.94, 5.88, 11.76, 14.7, 29.4 µM. The Mature embryos responded by swelling and callusing after 5 days of inoculation. Callus formed on all treatment was different in its texture and appearance. Exogenous supplementation of $AgNo_3$ enhanced the production of embryogenic callus. Maximum amount of embryogenic callus was formed on medium supplemented with $AgNo_3$ (11.76 µM) was transferred to modified regeneration medium with $AgNO_3$ (11.76 µM). Improved regeneration efficiency was also observed from the callus induced on $AgNO_3$ supplemented medium with it was transferred to normal regeneration media. Higher levels of $AgNO_3$ in regeneration medium distorted the levels and stems but it was tolerable upto 29.4 µM with decreased response for callus induction and plant regeneration.

Callus cultures:

The callus induced from different explants was further studied for its morphogenetic potential. Regeneration potential was explored for callus derived from different plant growth regulators and growth adjuvants.

Study of regeneration potential:

The primary callus induced from mature embryos, immature embryos cultured on MS medium supplemented with 2,4-D (0.5-5.0 mg/l) + Kn (0.5-2.0 mg/l) was

subcultured on MS medium supplemented with 2,4-D (0.5-2.0 mg/l) + Kn (0.5-1.0 mg/l). Loose, watery, translucent, non-embryogenic callus was discarded and only compact, nodular shiny morphogenic callus was subcultured on MS medium supplemented with 2,4-D (2.0 mg/l) + Kn (0.05 mg/l). The subcultured callus proliferated well and amount increased within three weeks. Embryogenic callus with green and nodular and shiny structures was subcultured at 2-3 weeks interval. Approximately 200-300 mg of callus was inoculated in each subculture for further maintenance and growth of callus.

Lower concentrations of Kn (0.05 mg/l) made a drastic increase in fresh weight of callus with each combination of 2,4-D. It was maximum on medium containing 2,4-D (2.0 mg/l). Higher concentrations of Kn led to emergence of shoot buds in callus cultured. The hard, compact, nodular callus subcultured on media supplemented with 2,4-D (2.0 mg/l) + Kn (0.05 mg/l) proliferated and became dark, green, compact along with production of watery, translucent and non-embryogenic callus. Early subculture and removal of non-embryogenic callus were found promontory for callus growth. The fresh weight and morphogenic potential of callus decreased if subculturing continued after 3 weeks. Callus showed browning of media with necrosis of callus.

Morphogenic potential and regeneration capacity of the callus was studied by subculturing of callus on MS media supplemented with Kn (2.0 mg/l) + IAA (0,5 mg/l). The callus subcultured on media supplemented with 2,4-D (2.0 mg/l) and Kn (0.05 mg/l) showed regeneration caapcity, higher concentration of Kn (1.0 mg/l) was not found promontory and callus lost its regenerability with further subcultures.

Callus could be maintained in morphogenetic condition of 3-4 subculture passage, however, with every subculture, fresh weight and regeneration potential decreased gradually.

Effect of growth adjuvants:

Various growth adjuvants viz., Glutamine (50-150mg/l) Ascorbic acid (10-30mg/l), Yeast extract (50,- 150 mg/l). AgNO3 (1,3,5 mg/l). Activated charcoal (10-30mg/l), PVP, (50, -150 mg/l) were added of MS medium containing 2,4-D (2.0 mg/l) + Kn (0.5 mg/l) for growth and morphogenesis in callus cultures. Growth was recorded best on $AgNo_3$ (3.0mg/l), ascorbic acid (30mg/l) but in appearance it looked like unorganized callus. Addition of PVP (100 mg/l) and Glutamine (150 mg/l) dramatically improved the growth and proliferation of callus

In Vitro Regeneration Pathways:

The present study was undertaken to trace out the higher frequency plant regeneration pathway in *Sorghum bicolor* and *Pennisetum glaucum*. After a comprehensive experimental study it was observed that when mature embryos were cultured on MS medium supplemented with different plant growth regulators, first embryos germinated and then callused. De novo meristemoid formation and their enlargement into hard globular structure was observed in sorghum bicolor. Along with the enlargement of meristemoids, organogenic callus formation was also observed. The small meristemoids turned into gaint meristemoids within three weeks. These gaint meristemoids were maintained by continuous subculture of 3 weeks interval on maintenance media. Serveral secondary de novo meristemoids developed on the outer periphery of the subcultured primary meristemoids. These gaint, green, shiny

meristemoids were transferred to regeneration media, where a large number of shoot buds emerged on outer periphery of these enlarged meristemoids. These shoot buds gave rise to healthy plantlets within 4 weeks under suitable culture conditions. The number of shoots produced through the enlarged meristemoids was very high which was advantageous for high frequency plant regeneration.

Rooting of Excised Shoots:

Plant regenerated on MS medium supplemented with Kn (2.0 mg/l) + IAA (0.5 mg/l) in sorghum and with Kn (0.5mg/l) + (IAA 1.0mg/l) in pennisetum were provided with weak and short roots. These roots were unable to absorb nutrients efficiently from the soil and to hold the standing plant. The regenerated shoots were taken out from the test tubes. After separating the shoots and cutting minor roots, these in vitro regenerated shoots were transferred to half strength MS medium, full strength MS medium and on it's alternations supplemented with IAA (0.5 mg/l) with varied amount of sugars 1-3% (w/v). After 4 weeks a cluster of long, healthy roots was observed arising from the basal node of the plant. Highly efficient root system that could support the plant under field conditions was developed on the half-strength MS medium supplemented with IAA (0.5 mg/l) and sucrose 3 % (w/v) in sorghum where as half strength of MS medium with 3% (w/v) sucrose was found suitable for raising efficient root system in pennisetum.

Field Transfer of in Vitro Plants:

In vitro regenerated plantlets with efficient root system were transferred to the pots containing 1:1 ratio of garden soil and organic manure. The pots were kept in protectd cage but totally under field conditions. No acclimatization was required prior to

transfer of the plantlets. All plantlets successfully grew and after 62-68 days fertile spike emerged from the plant. Many in vitro regenerated plant were grown to obtain the crop. .

DISCUSSIONS:

The major millets occupy an important place in food grain and forage for animals. During past few decades considerable efforts have been made for improvement of these plants but the in vitro response has been confined to only some varieties and genotype dependent. Present study was undertaken to establish healthy in vitro cultures with high regeneration capacity in sorghum and pennisetum. The composition of nutrient medium, selection of suitable explants and optimization of nutrient concentration have been considered important parameter to determine the morphogenic nature of callus and regeneration potential. Various nutrient media have been used for routine tissue culture of plants. The MS medium (1962) developed for tobacco has been found equally important for tissue culture of major millets. In vitro growth largely relies upon the addition of plant growth regulators but culturing the explants on proper nutrient medium may substitute the requirement of plant growth hormones (Ramage and Williams 2002, 2003). Although, the micronutrients are required in minute quantity but they play important role in plant tissue culture (Purnhauser 1991, Tahliani and Kothari, 2004, Chajer et al., 2008). The composition of culture medium plays a key role to determine the regeneration efficacy of cultures. The culture medium incorporated with a suitable carbon source in the form of sucrose is an essential requirement to establish the morphogenic cultures in major millets. The sucrose not only serves as carbon source but it also determines competence of morphogenic calli. The sucrose is commonly used carbon source in tissue culture of

millets but maltose is also used in pearl millet (‘O’ Kennedy et al., 2004; Tadesse et al., 2009; Gugsa and Kumlehn, 2011).

The importance of levels of auxins and cytokinins is another significant factor in establishment of efficient in vitro culture system for callus induction, organ formation and multiplication. In the present study different plant growth regulators were utilized in various concentrations. 2,4-D was found most effective among the auxins. It is the most commonly used auxin which has striking effects in tissue culture (Yao and Krikorian, 1981; Sears and Deckard, 1982 Wernicke and Brettell, 1982; Vasil, 1987; Mikami and kinoshita, 1988; Bregitzer et al, 1989; Akashi and adachi 1992a). Many cereal explants express embryogenic competence in the presence of 2,4-D. Adquate levels of 2,4-D help in perpetuation of embryogenecity of cultures. Lower to moderate levels of 2,4-D gave good response for callus induction and long term propagation. The different explants tired responded best at 2,4-D (0.5-2.0 mg/l) for morphogenic callus induction as also reported by previous workers (Bhaskaran and Smith, 1989; Nguyen at at. 1989; Cai and Butler, 1990; Maralappanavar et al. 2000; Seetharam et al., 2000). The callus induced on auxin supplemented media was of two types viz., unorgeanised, tranluscent, watery and organized with hard and nodular shiny structures. Such type of callus morphotypes have also been reported in many careals (Cai and Butler, 1990; George and Eapen, 1988; Kuruvinashetti et al., 1998; Raghvandra Rao et. Al., 2000) Higher concentrations of 2,4-D revealed a negative response. This is in conformity with early findings in sorghum (Wang and Vasil, 1982; Boyes and Vasil, 194; Cai and Butler, 1990). In the present study cytokinins alone and their combinations with auxins were also tested for induction of embryogenic callus. Cytokinins from lower the

higher levels could support germination only. It was observed that 2,4-D and pCPA in combination with Kn/BAP greatly favored the induction and proliferation of embryogenic callus from different explants. Similar response from seeds (Bhaskaran and Smith, 1989). Mature embryos (Thomas et al., 1977; Elkonin et al., 1984; Bhaskaran et. al, 1987; Mackinnon et al., 1987; Hagio, 1994;), immature embryos (Oldach et nal., 2001; Hagio 2002), shoot tip (Seetharam et al., 2003) and immature inflorescence (Cai and Butler, 1990) were reported.

The role of cytokinin in conferring competence is not clear. The use of cytokinin in sorghum callus cultures has been discouraged because of its negative influence on callus growth (Mastellar and Holden, 1970; Wernicke and Brettell, 1982). However, low levels of cytokinins were known to promote shoot out growth in sorghum cultures (Wernicke et al., 1982). Several investigators have used low levels of cytokinins in callus induction medium (Bhaskaran and Smith, 1988; Bhaskaran et al., 1983; Brettell et al., 1980; Cai et al., 1987; Dunstan et al., 1978; Ma et al., 1987; Mackinnon et al., 1987; Seetharam et al., 2000; Oldach et al., 2001; Hagio, 2002). Many reports suggest that successful regeneration was obtained on only cytoknin supplemented medium in pearl millet (Mythili et al., 1997;Goldmann 2003; Satyavathi 2006) On the other hand several investigators indicated that plant growth regulators are not essential to regenerate shoots in pearl millet (Campos et al.,2009)

In the present study mature and immature explants were used for callus induction and plant regeneration. The developmental stage has been found to be a critical factor in the establishment of totipotent cultures (Vasil, 1987). Mature explants in which cells have passed out the meristematic phase into a highly differentiated stage do not from

embryogenic callus as they are already committed to a particular development pathway (Morish et al., 1987). Immature embryos have been found best explants for raising embryogenic callus cultures, but availability of immature embryos is restricted to a particulars season of year whereas mature explants are available throughout the year. In the present study among the mature explants, mature embryos were found responsive for induction of embryogenic callus on medium supplemented with 2,4-D or pCPA in combination with cytokinins. Callus growth was observed from mature embryos cultured on medium with 2,4-D (2.0mg/l) and Kn (0.5 mg/l). The induction of embryogenic callus was also obtained on the medium supplemented with pCPA and pic. Embryogenic callus derived from different mature and immature explants was cultured on plain MS medium and medium supplemented with various concentrations of auxins and cytokinin viz, Kn, BAP, NAA, GA_3, IAA and IBA. Plantlet formation was also observed on plain MS medium without any plant growth regulators but regeneration response was poor in terms of average number of regenerants per callus culture. Best plant regeneration could be achieved on the regeneration medium supplemented with Kn (2.0 mg/l) and IAA (0.5mg/l) for sorghum and Kn (0.5mg/l) plus IAA (1.0mg/l) for penniseum.. Though the plant regeneration in major millets has also been reported on various media and combinations of auxins and cytokinins viz, MS basal medium and MS + BA (Raghvandra Rao et al., 2000; Kuruvinashetti et al, 1998; Mishra and Khurana, 2003), MS + Kn (Cai et al., 1987; George and Eapen, 1988; Cai and Butler, 1990_, MS + Kn or BA (George et al., 1991; Hagio, 2002), MS + BAP (Seetharam et cl., 2000; Saradamani et al., 2003), MS + Ba + NAA (Maralappanavar et al., 2000), MS + Kn + IAA or IBA (Sharma et al., 1989; Elkonin and Pakhomova, 2000), LS medium + BAP + IAA (Duncan et al., 1995), MS +

Kn or BAP and MS + BAP + NAA (Rao et al., 1995), MS + Kn + BAP (Sairam et al., 1999) and L_3 medium and L_3 + BAP (Oldach et al., 2001). In this study GA_3 and NAA could not improve the regeneration efficiency but they exhibited rhizogenesis in callus cultures. GA_3 alone and in combination with Kn has been used previously to promote the plant regeneration in Zea mays (Lu et al., 1982), *Eleusine coracana* (Kumar et al., 2001), *Pennisetum glaucum* (Lambe et al., 1999), *Paspalum dialatum* (Akashi and Adachi, 1992a) and Paspalum notatum (Chen et al., 2001b; Smith et al., 2002).

Multishoot formation or microtillering as a result of proliferation of axillary buds was observed when mature embryos were cultured on medium supplemented with NAA (0.5-10 mg/l) alone and in combination with Kn (0.5-1 mg/l) Microtillering has been observed in sorghum (Dustan et al., 1978, 1979), wheat (Rao and Kothari , 1992) barley (Kachwah and Kothari, 1994a,b) and finger millent (Vishnoi and Kothari, 1995) Reports are available on plant regeneration from mature embryos in cereals (Chin and Scott, 1977; Mohmand and Babors, 1990, 1991; Rao and Kothari, 1992; Oka et al., 1995; Varshney et al., 1999). Mature seeds and embryos have also been studied in millets but they produce lower amount of somatic embryos than immature embryos in kodo millet and pearlmillet (Vikrant and Rashid 2002b; Goldmann et al., 2003; Campos et al 2009).

In the present investigation leaf bases from in vitro grown seedling of different ages were used as explants to study the morphogenic potential. Low to moderate amount of callus could be induced but no plant regeneration was observed. In leaf base cultures of sorghum, Mishra and Khurana (2003) reported genotype dependent somatic embryogenesis and plant regeneration. Leaf bases have also been used as explants in Paspalum notatum (Shatters et al., 2994; Chen at al., 2001) orchard grass (Conger et al.,

1983), Kodomillet (Samantary et al., 1995, 1997) sorghum (Wernicke and Brettell, 1980, 1982, Wernicke at al., 1982, Cai et al., 1987), wheat (Chug and Khurana, 2003).Shoot tips in sorghum (Bhaskaran and Smith1992; Seetharam et al., 2000; Maheswari et al., 2006). Among the different explants used, immature embryos were found highly responsive under in vitro conditions in sorghum (Gamborg et. al., 1977; Thomas et al., 1977; Dunstan et al., 1978; Ma et al., 1987; Sharma et al., 1989; Lusardi and Lupptto, 1990; Rao et al., 1995; Kuruvinshetti et al., 1998; Elkonin and Pakhomova, 2000; Oldach et al., 2001; Hagio, 2002, Zapata, 2004). Callus induced from immature embryos cultured on medium supplemented with auxins and cytokinins was highly morphogenic. A combination of 2,4-D (2.0 mg/l) and Kn (0.5 mg/l) was found most effective for the embryogenic callus induction as previously reported in sorghum (Oldach et al., 2001; Hagio, 2002). 2,4-D supplemented medium has also been found suitable for callus induction and Proliferation in *Triticum aestivum* (Ozias-Akins and Vasil, 1982), *Pennisetum americanum* (Vasil and Vasil, 1981a, 1982b,; Campos et al., 2009; Jha et al 2009) *Panicum maximum* (Lu and Vasil, 1982), *Echinocloa frumentacea* (Talwar and Rashid, 1989), *Eleusine coracana* (Kumar et al., 2001). Other auxins such as pCPA, pic and 2,4-5-T were also found to be effective for callus induction from immature embryos in sorghum and pennisetum (Srivastav and Kothari 2002; O Kennedy etal.,2004; 2011a,b).

Embryogenic callus cultures in cereals contain two different morphotypes differing by morphology, texture, growth rate and extent of embryo differentiation. Long term retention of regenerability in these cultures is affected by several physical and chemical factor, which control the growth of tissues or cell grown under in vitro condition.

Browning of media because of phenolics in sorghum is associated with decreased in vitro morphogenesis (Gamborg et. al., 1977; Dunstan, 1978) Lusardi and Lupatto (1990) also reported the release of phenolic compounds in the medium, which affect the callus growth and regeneration potential. The regenerability in cultures could be maintained up to some extent by early subculturing the embryogenic callus on fresh medium. The embryogenic callus derived from different explants of sorghum could be maintained on medium containing 2,4-D (2.0 mg/l) and Kn (0.05 mg/l). Maralappanavar et al. (2000) reported the maintenance of embryogenic callus on medium supplemented with 2,4-D and Kn. However, Srivastav and Kothari, (2000) observed long-term maintenance of embryogenic callus on pCPA supplemented medium in pearlmillet.

Blackening and loss of vigor have been found obstacles to maintenance of embryogenic callus (Cai and Butler, 1999). In the present study exogenous supplementation of glutamine and PVP amd glutamine could increase in maintencance period. During each passage proliferated callus was tested for its regenerability after 2-3 weeks of every subculture. This is in agreement with the observations of Hagio (2002) that PVP is effective in reducing or preventing the damages caused by phenolic compound release in medium.

Cultures initiated from immature embryos of oats (Cummings et al., 1976), maize (Green and Philips, 1975) Pearl Millet (Vasil and Vasil, 1981) retained totipotency till 16 months. Saradamani et al., 2003, could propagate the embryogenicity for four months whereas, we could maintain the morphogenetic potential of callus culture upto nine months with exogenoun supply of glutamine and PVP to maintenance media. Adition of Glutamine promoted somatic embryogenesis in wheat (Ozias-Akins and Vasil, 1983a;

Redway et al., 1990; Vasil et al., 1992) and maize (Kamo et al., 1987). Proline is reported to enhance somatic embryogenesis in maize (Armstrong and Green, 1985; MacCain and Hodges, 1986; Suparasanna et al., 1994) Barley (Rengel and Jelaska, 1986), rice (Ozawa and Komamine, 1989). Tryptophan Promoted somatic embryogenesis in rice (Siriwardana and Nabors, 1983) in finger millet(Yemets et al.,2003).

We observed that higher levels of $CuSO_4$ in MS medium could greatly improve the efficiency of in vitro growth of callus and subsequent plant regeneration. The exact basis for this response in unknown but it has been suggested that 1-25 µM copper sulphate in a hydroponic nutrient solution exhibits the formation of ethylene precursor, 1-aminocyloporpance-1carboxy acid in rice (Lidon et a., 1995) and thus promote regeneration. Purnhauser and Gyuali (1993) suggested that it is not through the ethylene inhibiting action that copper ions promote regeneration since, cu+ ions are components or activators of many enzymes involved in electron transport, protein and carbohydrate biosynthesis and polyphenol metabolism. Optimized levels of copper lead to improved plant regeneration from callus cultures of barley (Bregitzer et al., 1998, Castillo et al., 1998, Chauhan and Kothari 2004) in rice(Sahrawat and Chand, 1999) *Capsicum annum* (Joshi and Kothari, 2007).

The role of zinc has not been fully investigated. He et.al, (1991) reported that there was marked increase in the formation of white structure on medium without zinc. These white structure have been described as embryogenic callus (Ozias-Akin and Vasil, 1983 a,b). The absence of zinc seems beneficial for somatic embryogenesis in wheat. Kothari et. al (2004) reported the influence of zinc sulphate for callus induction and plant regeneration in *Eleusine coracana*. They observed best callus induction of medium devoid of zinc

sulphate and best plant regeneration from the callus on normal level of zinc sulphate into regeneration medium. Kumar et al (2003) also evaluated the effect of zinc in Tinospora cordifolia and demonstrated that higher concentrations of zinc induce poor growth coupled with yellowing of leaves and stem. Higher concentrations of zinc inhibited cell division (Davies et al., 1991) and cell elongation (Godbold et al., 1983) and caused chlorosis of young leaves (Marschner, 1986). In contrast zinc deficiency decreases auxin concentration since zinc plays an important role in the synthesis of tryptophan a precursor of IAA. Zinc is also involved in protein synthesis, phytohormone activity, enzyme activation, membrance integrity and detoxification of superoxide radicals (Welch, 1995).

Iron is an important trace element. There is a critical requirement of iron for prolonged growth of plant tissue under in vitro conditions. He et al., (1991) reported that omission of iron from the medium significantly decreased shoot formation from embryogenic callus in Wheat. Iron is mainly present in the medium bound to the chelator Na2EDTA. A suitable from of iron has been found to be important for barley and wheat cultures (Novotny et al., 2000). Dahleen and Bregitzer (2002) reported that lower level of Fe_2 $(SO4)_3$ could significantly increase regeneration in barley. Chauhan and Kothari (2004) reported enhanced plant regeneration in rice with optimized levels of Fe_2 $(SO_4)_3$ and Na_2EDTA. The exact mechanism of involvement of iron for enhancing the in vitro morphogenetic response is not known but iron has been considered as an essential element and it probably enhance the morphogenesis through the enzymes containing iron. The enzymes contatining iron are involved in several biochemical reactions such as photorespiration, glycolysis, polymerization of phenols, redox reaction etc.

In the present investigation we observed the stimulatory effect of silver nitrate and Nickel chloride both for callus induction and plant regeneration. Silver nitrate has been shown to promote regeneration in several monocotyledonous plants such as wheat (Purnhauser et al., 1987), maize (Songstad et al., 1988), *Vanilla plamfolia* (Girdhar, 2001) in kodo millet and *Elusine coracana* (Chajer et al., 2008). Ghaemi et al., (1994) reported beneficial effect of silver nitrate on the production of embryoids from anthers of tetraploid wheat. The addition of silver thiosulphate, an ethylene antagonist has also been reported to stimulate plant regeneration in barley anther culture (Evans and Batty, 1994). Silver nitrate which is an ethylene inhibitor has also been reported to stimulate embryo production in wheat anther cultures (Lasherms, 1992). Our observations for beneficial effect of silver nitrate in sorghum is an further agreement with earlier finding in maize (Vain et al 1989) Elitriby et al., 2003) pearl millet (Oldach et al., 2001; Piue et al., 1983; Chajer et al., 2008). The role of $AgNO_3$ as suggested by Beyer (1976, 1979) was that ag+ inhibited the ethylene action by interfering with ethylene incorporation as its receptor site. Rouston et al. (1989) observed the promotive effect of cobalt and nickel on somatic embryogenesis in carrot. Nickel has been beneficial to avoid the avoid the metabolic stress and necrosis in tissues. Nickel is a cofactor for ureases enzyme ubiquitous inplants to hydrolyse urea (Gerenedas et al., 1999).

Micronutrients are essential elements taking part in redox reactions, structure and configuration of several enzymes and nucleic acid metabolism. Alterations in concentrations of the micronutrient viz., cobalt, manage and molybdenum could not make striking effect on in vitro morphogenesis. Thus, the overall discussion about the heavy

metals led to a conclusion that the levels of micronutrient in MS medium are not at their optimal level for enhanced in vitro morphogenetic response in sorghum.

Somatic embryogenesis has been proposed as the most common pathway of plant regeneration in tissue cultures of cereals and millets (Vasil, 1987; Kothari and Chandra, 1995; Campos et al 2009). Shoot morphogenesis from the exillary buds or adventitious shoot bud formation is also common in cereals (Lorz et al., 1988; Bhaskaran and Smith, 1990). There are several reports wherein both embryogenesis and shoot bud formation have been shown to occur in the same cultures in other ceareals (Lorz et al., 1988; Bhaskaran and Smith, 1990; Kothari and Chandra, 1995; Kothari and Varshney, 1998; Kaur and Kothari, 2004) in the present study we observed the formation of compact, hard nodular structure with shiny surface in cultures in sorghum. This is in agreement with early finding in sorghum (Sharma et al., 1989; George and Eapen, 1989). Earlier reports of plant regeneration in Sorghum described only somatic embryogenesis (George and Eapen , 1988; Bhaskaran and Smith, 1989; George et al., 1991; Seetharam et al., 2000; Arti et al., 1994; Raghvandra Rao et al., 2000; Mishra and Khurana, 2003). Organogenesis has also been reported as the mode of plant regeneration in sorghum (Sharma et al., 1989; George and Eapen, 1989). Hagio (2002) reported adventitious shoot formation in sorghum. Sairam et al., (2002 and 2003) developed efficient protocol for the induction of high frequency shoot proliferation in Tripsacum and corn. These shoots were shown to arise either directly by organogenesis or by somatic embryogenesis. The organogenesis is rare in millets. Finger millet and pearl millet were regenerated through organogenesis (George and eapen1990; Ceasar and Ignacimuthu 2008; Jha et al., 2009).