ANTHER CULTURE STUDIES IN RICE

(*Oryza sativa* L.)

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PAL-0218

DEPARTMENT OF PLANT BIOTECHNOLOGY

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ANTHER CULTURE STUDIES IN RICE

(*Oryza sativa* L.)

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PAL-218

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in

PLANT BIOTECHNOLGY

BANGALORE **JULY, 2012**


Affectionately
Dedicated to
My
Father Late
Shri Hari Ram

DEPARTMENT OF PLANT BIOTECHNOLOGY
UNIVERSITY OF AGRICULTURAL SCIENCES
BANGALORE-560065

CERTIFICATE

This is to certify that the thesis entitled "**ANTHER CULTURE STUDIES IN RICE (*Oryza sativa* L.)**" submitted by **Mr. DALPAT LAL, ID. No. PAL-218,** in partial fulfillment of the requirement for the degree of **Master of Science (Agriculture)** in **Plant Biotechnology** to the University of Agricultural Sciences, Bangalore, is a record of bonafide research work done by him during the period of his study in this University under my guidance and supervision and that no part of the thesis has previously formed the basis for the award of any degree, diploma, associateship, fellowship or other similar titles.

BANGALORE
JULY, 2012

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ANTHER CULTURE STUDIES IN RICE (*Oryza sativa* L.)

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THESIS ABSTRACT

Rice (*Oryza sativa* L., 2n=24), a member of the family Poaceae is the most important cereal food crop in the world and feeds over half of the global population. Anther culture is useful technique in rice breeding programs, as production of haploid plants speeds up the breeding cycle by fixing homozygosity in one generation. The investigation was conducted to standardize culture media for callus induction and regeneration from anther derived callus of Azucena (*O. sativa* subsp. japonica) and Budda (*O. sativa* subsp. indica) rice varieties. Anthers from the panicle, with a distance of 11cm and 8 cm length between the subtending leaf and the flag leaf in Azucena and Budda respectively, were selected for the study. At this length, anthers contained uninucleate pollen grains. Before inoculation of the anthers, a cold pretreatment of 5^0C for 8 days was given to the selected panicles. Callus induction frequency in different media combination ranged from 0.66% to 6.66% was observed in N6 medium supplemented with 1.0 mg l^{-1} 2, 4-D, 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} Kinetin. In Azucena variety, highest callus induction (6.66%) while no callusing was found in Budda variety. Highest shoot regeneration (0.33%) from callus was observed in MS medium supplemented with 0.5 mg l^{-1} BAP, 0.5 mg l^{-1} Kinetin and 80 mg l^{-1} adenine sulphate. But all the regenerated shoots were albinos. Root formation was observed in MS medium without any growth regulator.

Signature of the Student
(Dalpat Lal)

Signature of the Major Advisor
(Dr. Ashok T.H.)

ಭತ್ತದ ಬೆಳೆಯಲ್ಲಿ ಪರಾಗಕೋಶ ವಿಕಾಸದ ಅಧ್ಯಯನ

(*ಒರೈಸಾ ಸಟೈವ ಎಲ್.*)

ಅಮೂರ್ತ್

ಭತ್ತವು ವಿಶ್ವದ ಜಾಗತೀಕ ಜನಸಂಖ್ಯೆಯಲ್ಲಿ ಅರ್ಧಕ್ಕಿಂತ ಹೆಚ್ಚು ಜನರು ಬೆಳೆಯುವ ಪ್ರಮುಖ ಏಕದಳ ಆಹಾರ ಬೆಳೆಯಾಗಿದೆ. ಪರಾಗಕೋಶ ವಿಕಾಸದ ಭತ್ತದ ತಳಿ ಅಭಿವೃದ್ಧಿ ಯೋಜನೆಯು ಒಂದು ಉಪಯುಕ್ತವಾದ ವಿಧಾನವಾಗಿದೆ. ಅಗಣಿತ ಸಸ್ಯಗಳ ಉತ್ಪಾದನೆ ಒಂದು ತಲೆಮಾರಿನಲ್ಲಿ ಹೋಮೋಜೈಗೋಸಿಟಿ ಸ್ಥಿರೀಕರಿಸುವ ಮೂಲಕ ತಳಿ ಬೆಳೆಸುವಿಕೆಯ ಆವೃತ್ತಿ ಮೇಲೆ ವೇಗವನ್ನು ಹೆಚ್ಚಿಸುತದೆ. ಈ ಎಲ್ಲಾ ತನಿಖೆಯನ್ನು ಭತ್ತದ ತಳಿಗಳಾದ ಅಜೂಸಿನಾ (*ಒರೈಸಾ ಸಟೈವ ಎಲ್.* ಉಪಜಾತಿ ಜೆಪೊನಿಕಾ) ಬುಡ್ಡಾ (*ಒರೈಸಾ ಸಟೈವ ಎಲ್.* ಉಪಜಾತಿ ಇಂಡಿಕಾ) ಭತ್ತದ ಪರಾಗಕೋಶದಿಂದ ಕ್ಯಾಲಸ್ ಪ್ರವೇಶ ಮಾಡಿ ಮತ್ತು ಪುನರುತ್ಪಾದನೆ ವಿಕಸನಕ್ಕೆ ಮಿಡಿಯಾ ಪ್ರಮಾಣೀಕರಿಸಲು ಪರೀಕ್ಷೆ ನಡೆಸಲಾಯಿತು. ಒಂದು 11 ಸೆಂ.ಮೀ. ಮತ್ತು 8 ಸೆಂ.ಮೀ ಉದ್ದದ ಎಲೆ ಹಾಗು ಹೂಗೋಂಚಲಿನಿಂದ ರೋಪಿಸುವ ಭತ್ತದ ತಳಿಗಳಾದ ಅಜೂಸಿನಾ ಮತ್ತು ಬುಡ್ಡಾ ನಡುವೆ ಪರಾಗ ಅಧ್ಯಯನಕ್ಕೆ ಆಯ್ಕೆ ಮಾಡಿಕೊಳ್ಳಲಯಿತು. ಈ ಅಳತೆಯಲ್ಲಿ ಪರಾಗ ಏಕ ಬೀಜೀಕರಿಸುವ ಪರಾಗರೇಣುಗಳಿಂದ ಹೋಂದಿತ್ತು. ಪರಾಗ ರೋಗಾಣು ಚುಚ್ಚಿಕೆಯನ್ನು 8 ದಿನಗಳ ಕಾಲ 5°ಸೆ.ನಷ್ಟು ತಣ್ಣನೆಯ ಮೊದಲ ಚಿಕಿತ್ಸೆಯನ್ನು ಹೂಗೋಂಚಲಿಗೆ ನೀಡಲಾಯಿತು. ಆ ಸಂಯೋಜನೆಯಲ್ಲಿ ಕ್ಯಾಲಸ್ ಆವರ್ತನೆ 0.66% ನಿಂದ 6.66% ಮುಟ್ಟಿತು. N6 ಮೀಡಿಯಾದ ಸರ್ವೇಕ್ಷಣೆಯನ್ನು 1.0 ಎಮ್‌ಜಿ l^{-1}, 2,4-D, 2.0 ಎಮ್‌ಜಿ l^{-1} NAA ಮತ್ತು 0.5 ಎಮ್‌ಜಿ l^{-1} ಕೈನೆಟಿನ್‌ನಲ್ಲಿ ಮಾಡಲಾಯಿತು. ಇದರಲ್ಲಿ ಅಜೂಸಿನಾ ತಳಿಯಲ್ಲಿನ ಅತಿ ಹೆಚ್ಚು ಕ್ಯಾಲಸ್ ಪ್ರವೇಶವು (6.66%) ಬುಡ್ಡಾದಲ್ಲಿ ಕಂಡುಬಂದಿಲ್ಲ. ಕ್ಯಾಲಸ್‌ನ ಅತ್ಯಂತ ಉನ್ನತ ಚಿಗುರು ಪುನರುಶ್ಚೇತನಕ್ಕೆ (0.33%) 0.5 ಮಿಗ್ರಾಂ l^{-1} BAP, 0.5 ಮಿಗ್ರಾಂ l^{-1} ಕೈನೆಟಿನ್ ಮತ್ತು 80 ಮಿಗ್ರಾಂ l^{-1} ಅಡಿನೈನ್ ಸಲ್ಫೇಟ್ ಪೂರಕವಾಗಿರುವ ಎಮ್‌ಎಸ್ ಮೀಡಿಯಾದಲ್ಲಿ ಹಾಕಲಾಯಿತು. ಆದರಲ್ಲಿ ಚಿಗುರುಗಳು ಅಸಹಜ ಬಣ್ಣದಲ್ಲಿ ಇತ್ತು ಆದರಿಂದ ಬೇರುಗಳ ರಚನೆಯಲ್ಲಿ ಯಾವುದೆ ಬೆಳವಣಿಗೆ ನಿಯಂತ್ರಣವಿಲ್ಲದೆ ಎಮ್‌ಎಸ್ ಮೀಡಿಯಾದಲ್ಲಿ ವೀಕ್ಷಣೆ ಮಾಡಲಯಿತು.

ವಿದ್ಯಾರ್ಥಿಯ ಸಹಿ
(ದಲಪತ್‌ಲಾಲ್)

ಮುಖ್ಯ ಸಲಹೆಗಾರರು
(qÁ¦ C±ÆÃPï.n.JZï)

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INTRODUCTION

I. INTRODUCTION

Rice (*Oryza sativa* L., 2n=24) is one of the most versatile and important cereal crops of Poaceae family cultivated for more than 10,000 years (Sasaki, 2005). Over 90% of rice is produced and consumed in Asia alone. This accounts for about 29.3% and 29.9% of caloric intake in Asia and India respectively (*FAO Food Balance Sheet, 2007*). Rice is essentially a crop of warm humid environment and mainly grown under assured rainfall or irrigation. The area covered under rice in the world is 161.42 million hectares with a total production of 678.69 million tonnes and average productivity of 4204 kg per hectare (FAOSTAT, 2009). India has the largest area (44.1 million hectares) under rice among the rice growing countries of the world, but ranks second in total production. In India annual production of rice is 131.27 million tones with a productivity of 2977 kg per hectare (FAOSTAT, 2009).

Currently this crop supports more than 50% of the world population (Christou, 1997). Rice consumers are increasing at the rate of 1.8% every year. But the rate of growth in rice production has slowed down. It is estimated that rice production has to be increased by 50% by 2025 (Khush and Virk, 2000).

Rice biotechnology has already contributed to the production of improved varieties; Wide hybridization, including use of embryo rescue technique has allowed the transfer of useful genes from wild *Oryza* species to elite cultivars. Many more potentially useful genes remain available in the wild species and new tools of biotechnology will speed up their utilization. Protoplast regeneration protocols have become more efficient and available for a broader range of rice cultivars; Protoplast fusion followed by regeneration should become another technique for wide crossing.

The potential of plant tissue culture techniques for the development of novel plant genotypes has been increasingly recognized. Progress has been made in developing techniques to culture and regenerate plants from somatic cells, pollen and protoplasts of a large number of plant species (Thorpe, 1990). Tissue culture techniques coupled with the recent developments in molecular biology have opened a new vista for broadening crop gene pools, increasing the efficiency of conventional plant breeding methods.

Many new rice cultivars have been developed through biotechnological techniques like anther culture, embryo rescue and somaclonal variation (Brown and Thorpe, 1995; Zapata et *al.*, 2004).

The potentiality of microspores of cultured anthers to go through an embryogenic type of development was first demonstrated by Guha and Maheshwari (1964) in *Datura innoxia* (Solanaceae). One of the most striking examples of cellular totipotency is microspore or pollen embryogenesis (also referred to as androgenesis) where immature pollen or microspores are induced for the complete re-programming of the developmental plan, to embryo with the haploid (gametic) number of chromosomes rather than pollen grain formation (Reynold, 1997). Haploid plants have the genotypic number of chromosomes, that is a single set of chromosomes in sporophyte. Haploidy may be induced by different techniques, the most promising and successful one being microspore androgenesis. *In vitro* response of anthers of both japonica and *indica* are genotype specific and affected by too many factors. Thus each genotype needs standardization.

The technique has been used successfully to produce homozygous breeding lines in japonica rice (Brar and Kush, 2006). However, the potential for indica rice anther culture is yet to be fully exploited due to various constraints that include a recalcitrant genetic background in the

indica varieties (He *et al.* 2006). This technique comprises two steps: the induction of embryogenic callus from microspores followed by green plantlet regeneration from the embryogenic callus (Lentini *et al.*, 1995; Raina and Zapata, 1997).

The production of haploid rice plants by anther culture was first reported by Niizeki and Oono (1968). Subsequent studies have shown that the development of microspores into fertile plants depends on many factors, any of which can be limiting. These include plant genotype (Shen *et al* 1982), the developmental stage of the microspore (Chen 1976), cold pretreatment of the anthers (Sunderland 1978, Chaleff and Stolarz 1981, Chen *et al.,* 1982, Zapata *et al.,* 1982), growth conditions of the donor plants (e.g. photoperiod and light intensity (Lee *et al.,* 1988), the orientation of the plated anthers (Mercy and Zapata, 1987), and the nitrogen source of the callus-induction medium (Chen *et al.,* 1982, Tsay *et al.,* 1982).

Microspore or pollen embryogenesis (also referred to as androgenesis) is regarded as one of the most striking examples of cellular totipotency reported (Reynolds, 1997), but also as a form of atavism. This discovery paved the way for extensive research on anther culture that was particularly successfull in the Solanaceae, Brassicaceae and Gramineae. However, not all of the angiosperm crops of interest efficiently respond to embryogenesis induction; Although barley (*Hordeum vulgare*), rapeseed (*Brassica napus*), tobacco (*Nicotiana spp.*) and wheat (*Triticum aestivum*) are considered to be model species to study microspore embryogenesis due to their high regeneration efficiency (Forster *et al.*, 2007). Other scientifically or economically interesting species, such as Arabidopsis, many woody plants or members of legume family, still remain recalcitrant to this type of in vitro morphogenesis

(Sangwan *et al.*, 1986; Bajaj, 1990; Raghavan, 1990; Wenzel *et al.,* 1995; Germana, 2009).

In rice, anthers inoculated at mid to late uninucleate pollen stage have been found to be the most suitable for culture. Isolation and culture of microspores to respond to extrinsic factors necessary to initiate or enhance androgenesis. Studies have shown that several environmental cues may serve as stimuli for pollen embryogenesis.

Anther culture is a tissue culture technique which can be applied in plant breeding to accelerate the process of obtaining pure lines with numerous advantages: shortening breeding cycle by immediate fixation of homozygosity, which allows an easy selection of phenotypes for quantitative characters. Increased selection efficiency, widening of genetic variability through the production of gametoclonal variants, and allowing early expression of recessive genes. In addition, the screening of haploid cells against cold tolerance, salinity, pathotoxins and other biotic and abiotic factors before plant regeneration also becomes possible. Haploids are also valuable to detect and fix desirable recessive traits introduced through mutation (Chen *et al.*, 2001) or hybridisation (He *et al.*, 2006).

The present investigations were undertaken with the following objectives:

- Standardization of culture media for callus induction
- Regeneration from callus.

REVIEW OF LITERATURE

II. REVIEW OF LITERATURE

Rice is the world's single most important food crop and is the primary food for more than one third of the world's population. Production and consumption are concentrated in Asia where more than 90% of all rice is produced and consumed. Its importance can be estimated by the fact that the year 2004 was declared as International Year of Rice by the United Nations Food and Agriculture Organization. In direct proportion to the predicted rise in the world's human population, rice consumption and demand will increase over the next several decades (Kathuria *et al.,* 2007).

The rice cultivation has remarkable diversity because of its long history of cultivation and selection under diverse agroclimatic and biotic environments. Today rice is grown in more than 100 countries, extending from 53°N latitude to 40°S and from sea level to an altitude of over 3000 m.

Asian cultivated rice (*Oryza sativa* L.) consists of two major groups, which are known by the subspecies names *indica* (*O. sativa* ssp. *indica*) and *japonica* (*O. sativa* ssp. *japonica*). The *indica* subspecies is the most widely cultivated form of rice worldwide.

The relevant literature pertaining to the study is reviewed below under the following headings.

2.1 Plant cell and tissue culture

Plant cell and tissue culture is an important tool in both basic and applied sciences, including plant breeding. It also has vast number of commercial applications. Haberlandt in 1902 suggested the possibility of culturing artificial embryos from vegetative cells (Krikorian and Berquan, 1969). He enunciated the concept of 'totipotency', *viz.,* all the living cells

containing a normal complement of chromosomes could be capable of regenerating into entire plant. He visualized growing plant cells in artificial media to rejuvenate quiescent and differentiated cells, to trigger them into division and growth, forming tissues and regenerating into a mature plant. White (1934) first demonstrated the idea of Haberlandt by the successful culturing of excised roots of tomato. Further independent studies by Gautheret (1939) and others demonstrated that plant cells in culture can be made to proliferate continuously and also undergo differentiation.

Skoog and Miller (1957) demonstrated that regulation and differentiation of shoots and roots (organogenesis) in tobacco pith cultures depend on the relative concentrations of the auxin and cytokinin in the culture medium, thus introducing the concept of hormonal control of organ formation. Their results became the guiding principles of *in vitro* organogenesis. The phenomenon of somatic embryogenesis was first observed in suspension cultures of carrot by Steward and co-workers (1958) and in carrot callus grown on agar medium by Reinert (1959). By late 1970's it became evident that plant tissue culture technology was beginning to make significant contributions to agriculture and industry.

Asexual embryogenesis (Somatic embryos), was described in carrot by Steward *et al.* (1958), which provided a powerful technique for the mass production of artificial embryos. The possibility of obtaining *de novo* variation by plant tissue culture was reported in rice by Oono (1978). Tissue culture experiments in tobacco provided a standard medium for the successful *in vitro* cultures in a large number of Angiosperms (Murashige and Skoog, 1962). All these, among many other major experiments set start the intensive research programmes in plant tissue culture.

Cell and tissue culture technology is increasingly being applied for the genetic improvement of a wide range of Angiosperm species; cereals, legumes, root, fiber and sugar crops, oilseeds, tropical and subtropical fruit trees, wood species, and those yielding plant extracts. The species belonging to Poaceae family were considered recalcitrant to growth in culture, and hence were a neglected group. One major obstacle to the exploitation of cell and tissue culture techniques was the relatively unresponsive behaviour of these grasses (Maddock, 1985).

However, sustained efforts at *in vitro* culture of these species have yielded standard protocols in recent years. The advances in modern techniques of cell culture and molecular biology and the experience gained over the years in the field,have enabled successful application of modern methods of genetic improvement to the members of Poaceae family.

2.2 Normal male gametophytic pathway

Male reproductive processes take place in the stamens in flowering plants. The diploid cells undergo meiosis and produce haploid male spores or microspores. The completion of male meiosis marks the initiation of a unique pathway of cellular differentiation that leads to the formation of the male gametophytes - pollen grains - in flowering plants. This pathway is critically dependent on the asymmetric division of the microspore, termed pollen mitosis I, producing two unequally sized daughter cells, which have dramatically different structures and developmental fates. The large vegetative cell has dispersed nuclear chromatin, constitutes the bulk of the pollen cytoplasm and accumulates an abundance of stored metabolites (starch and lipids) required for rapid growth of the pollen tube. By contrast, the diminutive generative cell which has condensed nuclear chromatin, migrates into the cytoplasm of the vegetative cell and contains relatively few organelles and stored

metabolites. Whereas the vegetative cell exits the cell cycle, the generative cell completes a further, symmetrical mitotic division to form the two sperm cells (Figure 1) (Bedinger, 1992; McCormick, 1993).

2.3 Anther culture (Androgenesis)

Androgenesis in flowering plants is a unique biological process. Androgenesis is defined as a development route, alternative to zygotic embryogenesis whereby a haploid individual is obtained from a male derived haploid (reduced) nucleus. In haploids, induced mutations are readily detected and doubled haploids provide immediate homozygosity and the recovery of alleles not detected in heterozygous state. Hence, the production of haploids and doubled haploids in large number is of greater value to plant breeders.

Between 1940 and 1960, several techniques such as interspecific hybridization and pollination with irradicated pollen were developed for the induction of haploids. However, such methods were time consuming and gave haploids with irregular and low frequencies. Consequently the discovery of the fact that haploid embryos and plants could be obtained regularly and with relatively high frequency from *in vitro* culture of immature anthers of *Datura innoxia* by Guha and Maheshwari (1964), created considerable excitement among tissue culturists and plant breeders. Soon after this, Niizeki and Oono (1968) showed that a large number of normal haploid green plants could be obtained within a short time (5-7 weeks) by anther culture in rice. Since then induction and development of haploid calli, embryo and plants from anther/pollen culture have been well established in several species.

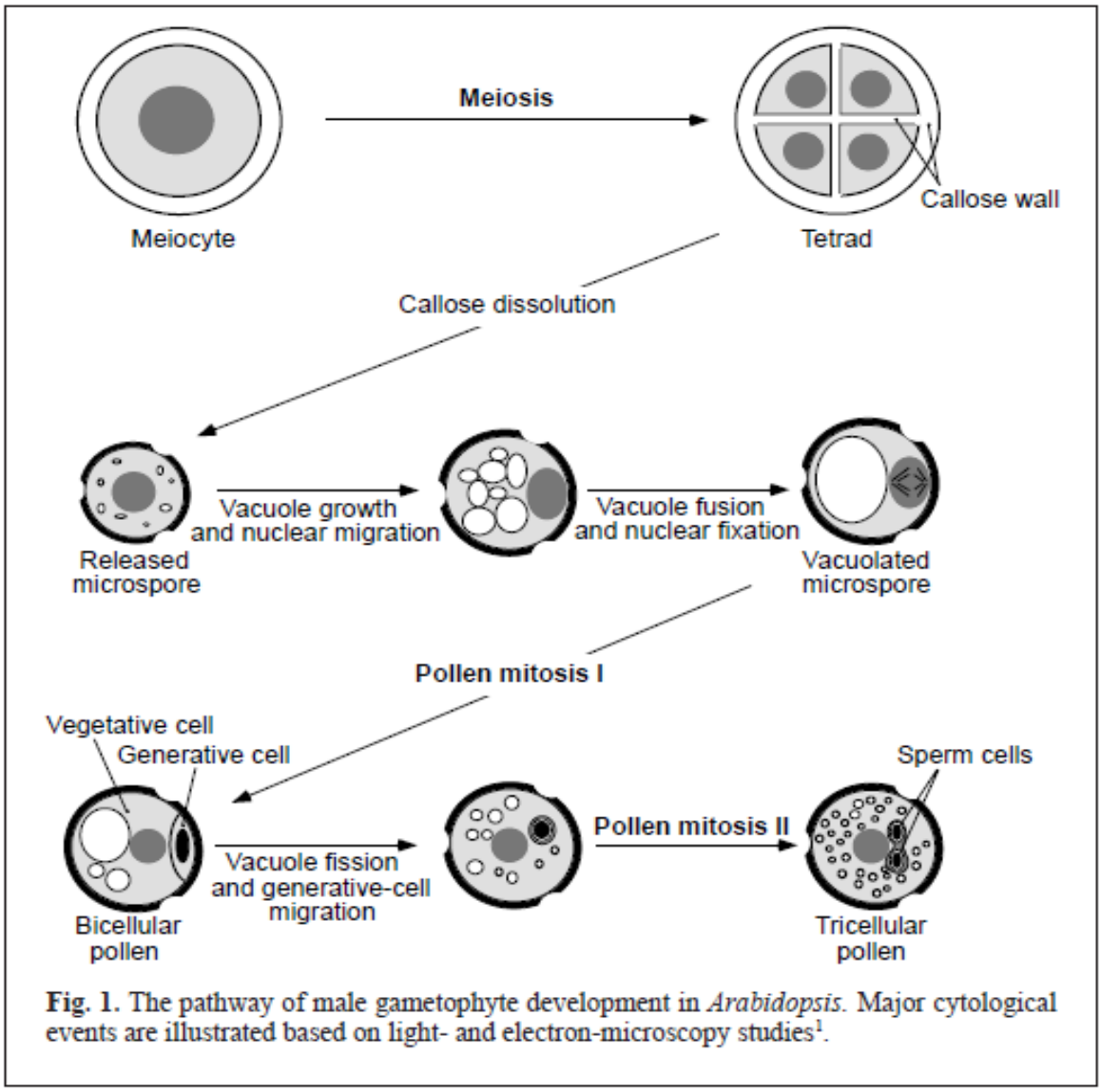


Fig. 1. The pathway of male gametophyte development in *Arabidopsis*. Major cytological events are illustrated based on light- and electron-microscopy studies[1].

Figure 1 : The pathway of male gametophyte development in *Arabidopsis*. Major cytological events are illustrated based on light and electron microscopic studies (David *et al*, 1998)

2.3.1 Pathways in pollen embryogenesis

Anther culture is the main technique for haploid induction in crop improvement. Pathways involved in pollen embryogenesis in *Nicotiana* and *Datura* was explained in more detail by Sunderland and Dunwell (1974), Reinert and Bajaj (1977), and Nitsch (1969). The two major pathways from pollen grains to plants are direct and indirect androgenesis (Figure 2). The direct pathway involves microspore differentiation through a series of stages that stimulate embryogenesis *in vivo* from the zygote. Globular stage embryos generally are released from the pollen grain wall, they develop through the heart and torpedo stages, and finally the cotyledons unfold and the plant emerges from inside the anther wall within 4 to 8 weeks. The indirect pathway involves the formation of a callus from the microspore, which bursts through the anther wall, and then differentiates to form either embryos or roots and shoots (organogenesis). The callus-derived plants are generally undesirable as they exhibit genetic variation and polysomy. Thus, the direct pathway is generally more desirable than the indirect one. This is especially true for any application of the technology in the context of genetic improvement.

Another alternative is to culture isolated or shed microspores. However the reports on isolated microspore culture are rather limited. In majority of the cases, *in vitro* response of microspores was observed within the anthers. Since the beginning of modern plant breeding practices, intensive efforts have been made to speed up the production of homozygous lines, which normaly requires at least six inbreeding generations Datta, (2005); Maraschin *et al.,* (2005); Germana, (2006); Pauls *et al.,* (2006); Shariatpanahi *et al.,* (2006); and Forster *et al.,* (2007) reviewed the different aspects of androgenesis induction, and potentialities.

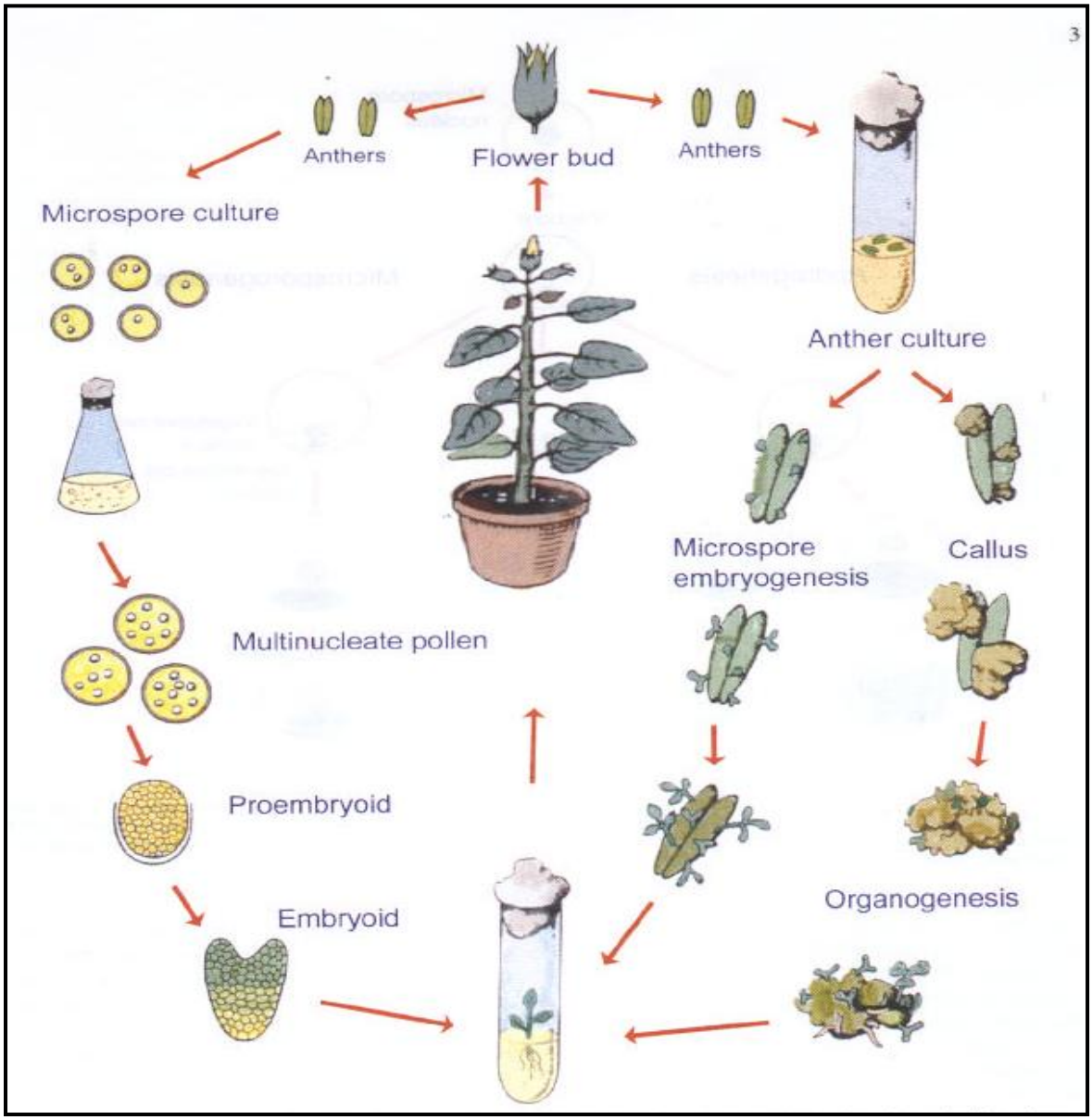


Figure 2 : Diagrammatic representation of the different modes of androgenesis and haploid plant formation by anther and pollen culture (Reynolds, 1997)

Three developmental patterns (Figure 3) involving the generative and vegetative nuclei in culture of pollen grains are recognized to be significant to pollen embryogenesis. Type A pattern involves repeated divisions of the vegetative nucleus until the exine ruptures, permitting the embryoid to continue its development inside the microsporangium. If the generative cell divides, it does so for a limited number of times and the cells formed do not contribute to the embryoid. In plants such as *Nicotiana tobacum* (Sunderland and Wicks, 1973), *Datura* (Vasil, 1980) and *Zea mays* (Barnabas *et al.,* 1987) the vegetative cell divides to produce the embryogenic precursor while the generative cell degenerates. Type B pattern involves a symmetrical division of the microspore into two equally diffuse nuclei, each resembling a vegetative nucleus. One or both of these nuclei can undergo further division and differentiation to form an embryoid. It is suspected that in some cases the two nuclei fuse to form a diploid embryo rather than the expected haploid condition (Vasil, 1980). Type C pattern involves a normal asymmetric division of the microspore into a generative and vegetative nucleus. This pattern differs from Type A, in that the generative nucleus contributes to the formation of the embryoid. Although this pattern has been observed only in *Datura itinoria,* it is suspected to be operative in other species in which nonhaploid embryoids are produced (Raghavan, 1976).

The A pathway of embryogenesis is characterised by an asymmetric first division followed by continued division of the vegetative cell (Al) and generative cell (A2) to form the proembryoid. The B pathway begins with a nonpolar division of the microspore producing two equivalent cells or nuclei, each of which contributes equally to the proembryoid.

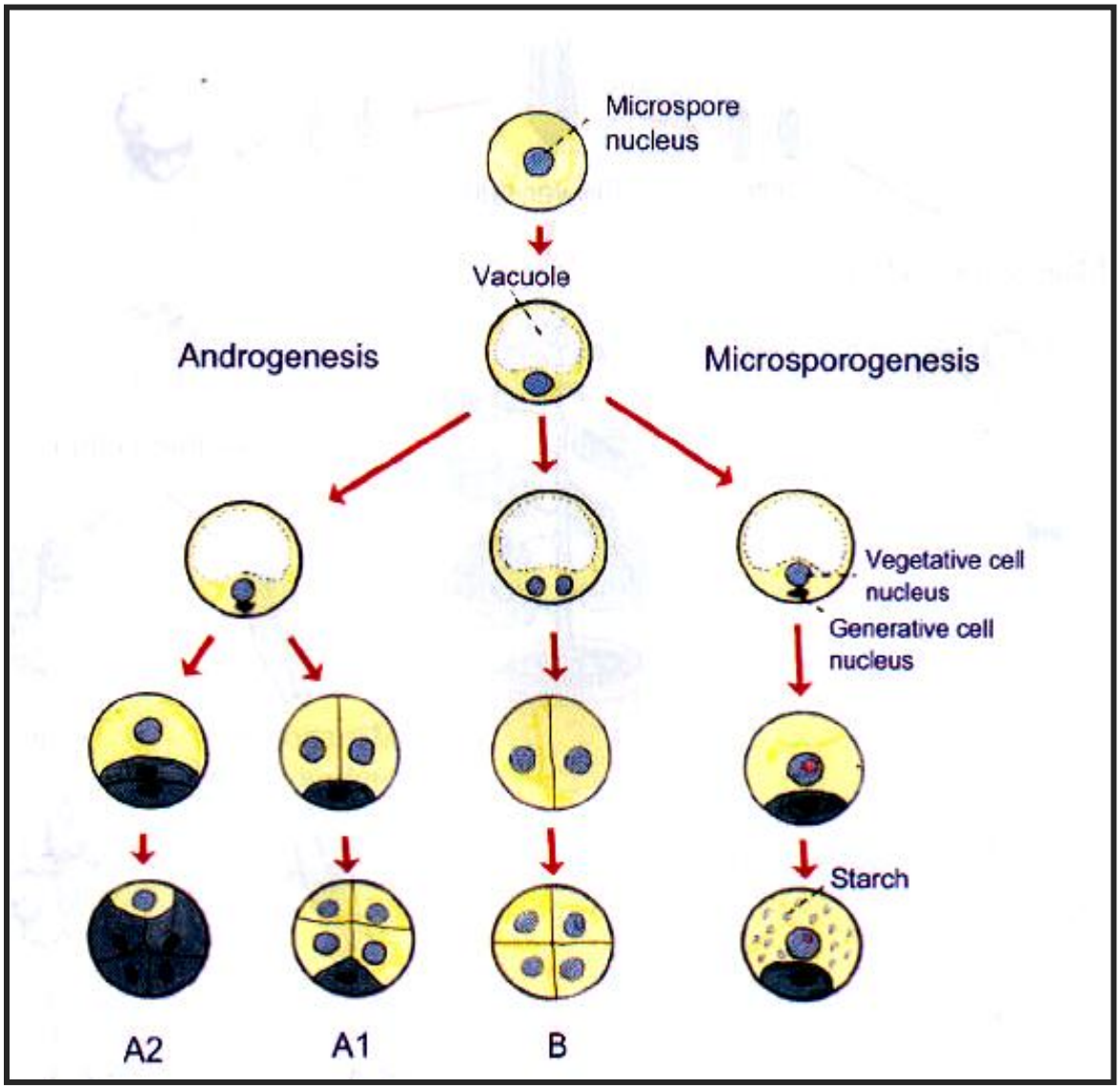


Figure 3 : Modes of microspore division during micro gametogenesis or induced embryogenesis (Sunderland, 1973)

Anther culture comprises of various steps starting from donor plant selection to callus induction and regeneration has been depicted through a flow chart in Figure 4.

2.4 Factors influencing androgenesis

Anthers containing pollen at the appropriate stage of development must be isolated from the donor plant for embryogenic induction to occur. Isolation and culture frees the microspores to respond to extrinsic factors necessary to initiate or enhance androgenesis. Studies have shown that several environmental cues may serve as stimuli for pollen embryogenesis.

The growth conditions (temperature, light and nutrient supply at the stage of anther formation) and physiological state of the donor plants at the time of explant collection is known to affect response in a number of plants (Karim and Zapata, 1993). The effect of the age of the parent plants, the stage of flowering and the position of the bud in the inflorescence indicate that the content of growth hormones in the growing tissues of the young bud may be relevant in this connection (Sun *et al,* 1993). Plants grown under natural light during the normal season have been found to be better than those grown under artificial light during offseason (Pandey, 1973).

Various factors influencing anther culture in rice is reviewed on following aspects.

2.4.1 Genotype

A genotype grown in a particular environment plays an important role in androgenic response. Many crop genotypes are quite recalcitrant in their *in vitro* response. It has been repeatedly reported that different cultivars within a species exhibit diverse responses in anther culture.

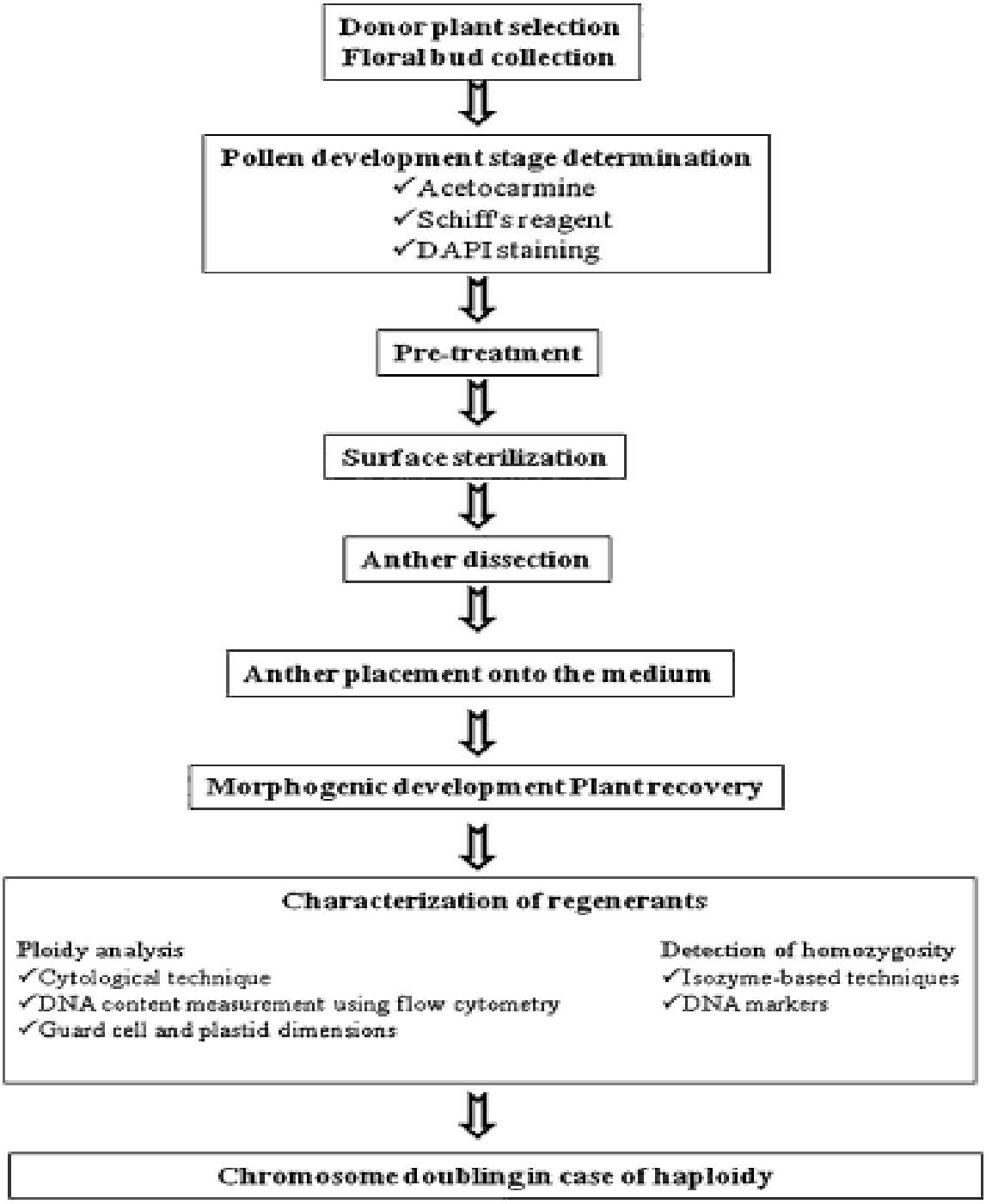


Figure 4 : Guidelines of common anther culture Method (Antonietta, 2011).

It is well known that induction frequency and regeneration ability of the pollen callus or embryoids vary greatly with the genotypes of the anther-donor plants. Several researchers like Niizeki and Oono (1968), Zapata (1985), Boyadzhiev and Kong (1989), Mikami and Kinoshita (1988), Quimio and Zapata (1990), and Iwai *et al.,* (1990) have observed genotype differences for culturability in rice.

Bajaj (1990) studied 21 cultivars of *Triticum aestivum*. Haploid tissue could be obtained from anthers of only ten cultivars, while in rice, japonica subspecies have been found to be more productive than indica subspecies.

Genotypic variation is mostly seen in the extent of response rather than in the existence of responsive and non-responsive genotypes. In general, *japonica* rice genotypes responded better than *indica* (Zapata, 1985; Manimekalai and Rangaswamy, 1983 and 1987; Yang, 1988; Narasimman *et al.*, 1990; Quimio and Zapata, 1990), and F_1 hybrids perform better than their inbred parents (Manimekalai and Rangaswamy, 1987). It was suggested that the pollen callus induction frequency is controlled by genes of diploid heterozygous anther wall tissue and not mainly by genes of the hemizygous microspores (Junwen, 1986). Genotypic variation to the extent of green plant or albino plant regeneration is also reported in rice (Datta *et al.,* 1990).

Japonica races of rice seem to be good for callus induction as compared to *indica* (Quimio and Zapata, 1990). Studies on the genetics of anther culturability revealed that this trait was heritable (Narasiman and Rangaswamy, 1993) and had independent inheritance (Davoyan, 1987), had no maternal influence (Quimio and Zapata, 1990), and had dominant gene action (Chen and Chen, 1993). On the other hand cytoplasm can significantly affect tissue culturability (Sagi and Barnabas, 1989).

Donor plants of wheat and barley grown during October–December provided an excellent microspore response (work at Grünbach, Germany, 1985–86). In the rice crop, plants grown during the dry season have provided the best microspore response (work at IRRI during 1993– 2000). The percentage of anthers producing microspore embryos and the number of regenerates produced per anther appear to be determined independently (Dunwell, 2010).

2.4.2 Ontogenetic age and physical condition of the donor plant

The physiological state of the donor plant at the time of explant collection is known to affect response in a number of plants (Karim and Zapata, 1993). Anthers from flower buds arising early with the onset of flowering are better than the buds arising later in the season (Narayanaswamy and Chandy, 1971; Sunderland, 1971). In rice, panicle-to-panicle differences in callus induction has been reported. Although during the incubation of anthers, light has not been found to have a crucial effect on callus induction, the light condition under which the parent plant is grown appears to have a considerable effect (Rangaswamy *et al.*, 1992). Plants grown under natural light during the normal season have been found to be better than those grown under artificial light during off-season (Pande, 1973). In rice, temperature and sunshine during flowering affected anther response markedly (Hu *et* al., 1978b). The indica rice Basmathi-370, gave a better response when on flowering the average maximum temperature range was 34.2 to 23.3°C (Raina *et al.,* 1987).

Lee *et al.*, (1988) reported that the rate of 'S' pollen grain *in vivo* and the callus formation efficiency *in vitro* were significantly increased in long day conditions than short day condition, and more increased in *Japonica* type of late ripening cultivars than in early ripening cultivars.

In wheat the most important conditions for androgenesis during growth of donor plant might be temperature, light and nutrient supply at the stage of anther formation (Carman and Jefferson, 1987).

2.4.3 Donor plant-culture treatments

Large improvements in embryoid formation could be made by improving environmental conditions. The effect of the age of the parent plants, the stage of flowering, and the position of the bud in the inflorescence indicate that the content of growth hormones in the growing tissues of the young bud may be relevant in this connection. Thus, hormones - IAA, NAA, or GA alone or in combinations sprayed on parent plant about one week before using the bud, may be beneficial for induction of pollen haploidy (Sun *et al.*, 1993).

The physiological state of the donar plant at the time of explant collection is known to affect response in a number of plants (Karim and Zapata, 1993). Anthers from flower buds arising early with the onset of flowering are better than the buds arising later in the season (Narayanaswamy and Chandy, 1971; Sunderland, 1971).

Plants grown under natural light during the normal season have been found to be better than those grown under artificial light during off season (Pandey, 1973).

The induction of androgenesis depended on the genotype of the donor plant, the stage of pollen development and the use of cold treatment. Anther response was 17- 88% depending on the genotype. The best induction of androgenesis was achieved with anthers extracted from flower buds 13-23 mm long after 24 h cold treatment at 4°C. The first haploids emerged 4 weeks after culturing on half-strength MS medium. When they reached a height of 15 cm they were transplanted from full-strength MS medium to *in vivo* conditions.

2.4.4 Pre and post inoculation physical environmental conditions

The pre and post inoculation treatments are given as a means of neutralizing the more powerful male potency normally present in the young microspores of the majority of flowering plants. During the cold-shock treatment, additional divisions of the non-gametophytic type are known to occur and these facilitate the induction process in the dedifferentiating culture medium (Nitsch, 1974).

In graminaceous crops, it has been reported that cold-shock pre-treatment of young spikes was effective for anther culture of *Oryza sativa* (Zhou and Cheng, 1982) including, other reveals. Among the *indica* and *Japanica* rice genotypes, the cold shock pre-treatment requirements varied (Zhou *et al.*, 1983). They also pointed out that when cold treatment duration exceeded a certain limit, the induction frequency decreased markedly. They also observed that cold treatment not only significantly increased anther response but also enhanced green plant production.

Shariatpanahi *et al.,* (2006) observed in many genotypes that physical or chemical pre-culture treatments applied to excised flower buds, whole inflorescences or excised anthers before culture act as a trigger for inducing the sporophytic pathway, thereby preventing the development of fertile pollen (gametophytic pathway). Protocols with special emphasis to widely used stress treatments on microspore embryogenesis in selected species are given in Table 1.

Pretreatments such as chilling, high temperature, high humidity, water stress, anaerobic treatment, centrifugation, sucrose and nitrogen starvation, ethanol, c-irradiation, microtubuli disruptive agents, electrostimulation, high medium pH and heavy metal treatment are particularly popular approaches in anther and microspore culture.

Table1 : Protocols with special emphasis on widely used stress treatments on microspore embryogenesis in selected species (Shariatpanahi *et al.*, 2006)

Plant	Stress	Protocol	Microspore stage	Result	System	Reference
Tobacco	Heat and starvation	33°C and B medium/6 days/ microspore suspension	Late UC	E&R	IMC	Touraev et al. (1996a)
Wheat	Heat and starvation	33°C and B medium/4 days/ anthers	Late UC-premitotic	E&R	IMC	Touraev et al. (1996b)
Brassica napus	Heat	32°C/72 h/microspore suspension	Late UC	E&R	IMC	Custers et al. (1994)
Broccoli	Heat	32.5°C/24 h/microspore suspension	Late UC-early BC	E&R	IMC	Dias (2003)
Barley	Starvation	Mannitol (0.7 *M*)/24°C/4 days/ anthers	Mid-late U	E&R	AC	Castillo et al. (2000)
Barley	Cold and/or starvation	+4°C and mannitol (0.3 *M*)/4 days/spikes +4°C/3–4 weeks/spikes mannitol (0.3 *M*)/25°C/4 days/ spikes	Mid-late UC	E&R	IMC	Kasha et al. (2001)
Barley	Cold	+4°C/2–4 weeks/spikes	Early-mid UC	E&R	IMC	Davies and Morton (1998)
Barley	Cold	+4°C/3–4 days/anthers	UC	E&R	AC	Jacquard et al. (2003)
Barley	Cold	4–5°C/21–28 days/spikes	Mid UC	C/E&R	AC	Szarejko (2003)
Wheat	Cold and/or starvation	+4°C and mannitol (0.4 *M*)/5–7 days/spikes +4°C/2–4 weeks/spikes mannitol (0.4 *M*)/25°C/5 days/ spikes	Mid-late UC	E&R	IMC	Kasha et al. (2003)
Wheat	Cold	+7°C/14 days/spikes	Early-mid UC	E&R	AC	Tuvesson et al. (2000)
Rice	Cold	8–10°C/8 days/panicles	Mid UC-early BC	C&R	AC	Zapata-Arias (2003)
Maize	Cold	+7°C/1 week/tassels	Mid UC	C/E&R	AC	Barnabas (2003b)
Triticale	Cold	+4°C/2 weeks/spikes	Mid-late UC	C/E&R	IMC	Pauk et al. (2000)
Triticale	Cold	+7°C/14 days/spikes	Early-mid UC	E&R	AC	Tuvesson et al. (2000)
Durum wheat	Cold	4°C/7 days/spikes	Mid UC	C/E&R	AC	Jauhar (2003)
Potato	Cold	4–6°C/72 h/buds	Late UC-early BC	C/E&R	AC	Aziz et al. (1999), Tai and Xiong (2003)
Apple	Cold and starvation	4°C/1–2 weeks/buds then 4°C and B med./2–3 days/microspore suspension	Late UC	E&R	IMC	Höfer et al. (1999)
Citrus	Cold	4°C/8–15 days/buds	Late UC	C&R	AC	Germana (2003)
B. napus	Colchicine	Colchicine (25 μ*M*)/42 h/25°C/ microspore suspension	Late UC	E&R	IMC	Zhao et al. (1996)
Wheat	Colchicine and mild heat	Colchicine (0.04%)/29°C/3 days/ anthers	Mid-late UC	C/E&R	AC	Barnabas (2003a)
Maize	Colchicine	Colchicine (0.02%)/3 days/ anthers	Mid UC	E&R	AC	Obert and Barnabas (2004)

UC: Uni-Cellular; B: Bi-Cellular; E: Embryo; R: Regenerate; C: Callus; IMC: Isolated Microspore Culture

In rice, different temperatures and incubation timings were found to give better results: 6°C for 5 days (Chaleff *et al.,* 1975); 10°C for 4-8 days (Hu *et al.,* 1978b); 5°C or 10°C for 7 or 10 days or 13°C for 10 or 14 days (Genovesi and Magill, 1979); 2-4°C for 48 hours (Cornejo Martin and Primo-millo, 1981); 7°C for 3 days (Chaleff and Stolarz, 1982); 9-10°C for 20 days (Zhou *et al.,*1983); 8°C for 4-8 days (Zapata *et al.,* 1983); 10°C for 10 days (Manimekalai and Rangaswamy, 1987); 10°C for 11 days (Gupta and Borthakar, 1987); 8°C for 8 days (Hue and Chae, 1987); 4°C and/or 10°C for 8-12°C for 8-16 days (Moon *et al.*, 1989); 10-18 days (Datta *et al.,* 1990); 6-8°C for 8 days (Quimio and Zapata, 1990); 4°C for 8 days (Reiffers and Adelson, 1990); 8^0C for 8 days (Afza *et al.*, 2000); 7-10^0C for 10 days. (Thuan *et al.*, 2001); 8^0C for 8 days (Gioi and Tuan, 2002); 6^0C for 7 days (Shahnewaz *et al.*, 2003); 4-8^O C for 8 days(Islam *et al.*, 2004); 8°C for 8 days (Roy and Mandal, 2005); 8±2°C for 7 days (Niroula and Bimb, 2009) ; 8°C for 7–10 days (Suriyan *et al.*, 2009); 8°C for 8 days (Nadali *et al.*,2009); 10^0C for 7days (Silva and Ratnayake, 2009); 4°C for 5 - 7 days.(Gueye and Ndoye, 2010); 8°C for 8 days (Abbasi *et al.*, 2011); and 7-10^0C for 5-10 days (Thanh and Nguyen, 2011).

Sen *et al.*, (2011) observed effect of cold pretreatment on callus induction and plant regeneration in anther culture of boro rice hybrids - Krishna Hansa x BPT 5204, Krishna Hansa x NDR 359, IR 64 x MTU 7029 and IR 64 x Jaya for various number of days. Callus induction and plant regeneration was significantly affected by the genotype and the length of cold pretreatment. Twelve days of cold pretreatment was most effective for callus induction in all the hybrids except IR 64 x MTU 7029.

Besides temperature, centrifugation of rice panicles at 2000 rpm for 10 min (Zhu and Wang, 1982) and irradiation of anthers prior to

culture (Zapata *et a1*., 1986) have been found to increase the callusing efficiency by more than 28 times.

2.4.5 Developmental stage of explant

2.4.5.1 Distance between the flag leaf and subtending leaf

Mercy and Zapata (1986), studied the distance between the flag leaf and the subtending leaf. Late uninucleate and early binucleate pollen stage has been used as markers for callus induction although with inconsistent success.

In rice, distance between the flag leaf and subtending leaf were found to give better results: Panicles were excised while still enclosed within the sheath (Gupta and Borthakur,1987); 3-6 cm (Reiffers and Adelson, 1990); 7–13 cm (Afza *et al.*, 2000); 5- 7cm (Thuan *et al.*, 2001); 3, 4, 5, 6, 7 and 8 cm (Silva and Ratnayake, 2009); 5-10 cm (Gioi and Tuan, 2002); 3-6 cm (Islam *et al.*, 2004); 5-9 cm (Nadali *et al.*, 2009); 7–10 cm (Suriyan *et al.*, 2009); 5 - 10 cm (Gueye and Ndoye, 2010); 4 to 8 cm (Abbasi *et al.*, 2011); and 5-10 cm (Thanh and Nguyen, 2011).

2.4.5.2 Pollen development stage

The stage of pollen development is usually tested in one anther per panicle by the aceto-carmine method (Gupta and Borthakur, 1987). Yin *et al.* (1976) using a *japonica* cultivar observed that the best microspore stage for callusing was the late- uninucleate stage when spikelets were yellowish green in colour and the length of stamen was 1/3 to 1/2 of the glume.

Chaleff and Stolarz (1981) reported that panicles harvested prior to emergence from the flag leaf sheath when the base of the flag leaf was 3-5 cm above the base of the next lower leaf, corresponds to late uninucleate stage of pollen in the anthers. Shahajahan *et al.,* (1992)

reported yellowish green colour of spikelets corresponds to mid uninucleate stage of the pollen where maximum response was observed.

In rice, anthers inoculated at mid to late uninucleate pollen stage have been found to be the most suitable for culture. Oono(1975), Chen and Lin (1976), Chen (1977) and Cornejo-Mart in and Primo-Millo (1981) conducted detailed studies. They concluded that anthers at the tetrad stage do not respond at all, and early uninucleate pollen may respond poorly. Mid to late uninucleate pollen respond the best. Anther response falls sharply after the first pollen mitosis. Anthers in early stages of nuclear division respond more readily than those in the later stages. Cal1i from late uninucleate pollen tend to show less regeneration potential and produce more albinos.

In rice, following stages of pollen were found to give better results; uninucleated stage (Reiffers and Adelson, (1990); Shahnewaz *et al.,* (2003); Islam *et al.*, 2004) and Silva and Ratnayake, (2009); mid-uninucleate (Gupta and Borthakur 1987); Nadali *et al.*, (2009); Afza *et al.,* (2000) and Gueye and Ndir, (2010); mid to late uninucleate (Thuan *et al.,* (2001); Niroula and Bimb, (2009) and Suriyan *et al.*, (2009); mid-uninucleate to early binucleate (Abbasi *et al.*, 2011); mid uni-nucleate to early bi-nucleate (Thanh and Nguyen, 2011).

Sopory and Munshi, (1996) reported that microspore stage would appear to affect the ploidy level of the plant produced in anther culture because, in their study, plantlets obtained from pollen at the uninucleate stage were found to be mostly haploids, whereas plants with higher chromosome numbers were produced by anthers at the later stages. A visual marker that shows good correlation with the pollen stage is used as a guide to identify the required stage of pollen during large scale culturing. Usually, the distance between the collar of the flag leaf and the

ligule of the penultimate leaf of the tiller serves as a reliable guide to anther maturity (Bishnoi *et al.*, 2000).

2.4.6 Spikelet position in panicle

Afza *et al.,* (2000) studied the effect of the spikelet position within the panicle on callus induction and plant regeneration. The potential of anthers from different parts of the panicle to induce callus was investigated with the *japonica* rice variety Taipei 309. The callusing abilities of anthers from different spikelet positions were significantly different. After plating 4483, 4496, 4348 anthers from the basal, middle and top parts, the percentage of anthers forming calli was 20% in the basal part, 12% in the middle part and 8% in the top part. The anthers of basal parts containing pollen at all uninucleate stages, including early, middle and late, showed higher callus induction frequency than those from middle and top parts. So the anthers from the basal part of the panicle should be used in anther culture of rice in order to obtain higher efficiencies.

2.4.7 Media and culture conditions

The nutrient medium not only provides nutrition to the microspores but also directs the pathway of embryo development. It is critical to change the composition of the media or replenish them to keep the balance of micronutrients and maintain the pH. The most commonly used basal media for anther culture are N6 medium (Chu 1978), (modified) MS medium (Murashige and Skoog 1962), Nitsch and Nitsch (1969) medium and B5 medium (Gamborg *et al.*, 1968), but there are many others. Generally, half-strength MS salt mixtures are suggested for the Solanaceae, and N6 medium for the cereals (Chu 1978).

Niizeki and Oono (1968) used Blaydes medium in their first report of successful rice anther culture. Until 1975 subsequent researchers used

the same formulation or attempted only slight changes. Oono (1975) examined several other media and recommended MS (Murashige and Skoog, 1962) medium as the most suitable medium for anther culture. Chu *et al.* (1975) reported a medium, N6 for rice anther culture. However, it is widely used for wheat anther culture.

Rice anthers respond to many basic media such as Miller (Wang *et al.*, 1974), modified MS medium (Chen, 1977), LS and modified LS media (Chaleff and Stolarz, 1981), and modified White's medium (Tsai and Lin, 1977). So far, N6 medium (Chu *et al.*, 1975) has been widely adopted for *Japonica* rice.

These authors pointed out that the growth and differentiation of rice pollen callus are influenced by major salts, especially by NH_4 salt. Lower concentration of NH_4 ion was beneficial. Boyadzhiev and Kong (1989) reported that N6 was better for callus induction in rice while MS was better for obtaining regenerations.

The most widely used medium for inducing higher anther response in japonica cultivars is the N6 medium (Chu, 1978) although the basal nutrients of this medium are not optimum for anther culture of indica rice (Lentini *et al.* 1995; Raina *et al.* 1989).

Many reports point out that media with a relatively high content of inorganic salts are more suitable for the differentiation of callus. The differentiation frequency of rice pollen callus on different media were as follows: Modified N6>MS>l/2MS>Miller>Nitsch. Addition of various concentrations of Na_2Fe-EDTA, Na_2Fe-EDTA, or a combination of these two compound improved plantlet production in rice (Chen *et al.,* 1986). Use of modified MS medium containing reduced nitrogen and 10 per cent (w/v) ficoll might favour microspore embryogenesis and green plant regeneration in rice (Datta *et al.*, 1990).

Two familiar basal media, the chemically defined N6 medium (Chu, 1978) and the MS medium (Murashige and Skoog, 1962), have been generally used with modifications. Anthers were floated on the surface of 10-ml aliquots of media in 50-mm sterile plastic dishes or 10–15 anthers were cultured in 24 wells containing 1.5-ml of the media. It has been established that the nitrogen composition of the culture medium plays a significant role in androgenesis (Raina and Zapata, 1997).

The nutrient medium not only provides nutrition to the microspores but also directs the pathway of embryo development. It is critical to change the composition of the media or replenish them to keep the balance of micronutrients and maintain the pH. The pH of the media, particularly liquid media, changes dramatically with time at the onset of embryo development (Datta and Wenzel, 1998).

Herath *et al.*, (2007) studied the optimization of media requirements and culture conditions for high frequency callus induction and plant re-generation of several indica × japonica F1 hybrids using improved anther culture media. Five indica rice varieties (BG 90-2, BG 379-2, BG 94-1, Dahanala and Suduru samba) and two japonica varieties (Hu Lo Tao and Chuan 4) were selected and F1 hybrids were taken with all possible crosses between them. Anthers were cultured in agar solidified modified N6, B5 and Millers media. Calli were transferred to MS medium for plant regeneration. Best callus induction frequencies (0.8 – 29.4%) were obtained in N6 medium containing 5% (w/v) sucrose, than B5 (0.5 – 19.2%) and Millers (0.2 – 19.8%) media. The F1 hybrids were more responsive to anther culture than their parents. Highest callus induction frequency of 29.4% observed in N6 medium for F1 hybrid Hu Lo Tao × BG 90-2. The green plant regeneration frequency of calli induced on N6 medium higher than the other two media and the highest frequency of 41.0% was occurred in F1 hybrids of Hu Lo Tao ×

BG 90-2. Modified N6 medium had positive effect on anther culture performance and the F1 hybrid Hu Low Tao × Bg 90-2 gave the best performance.

2.4.8 Concentration of carbon sources

A carbohydrate source is essential for embryo production in anther culture because of their osmotic and nutritional effects (Powell, 1990). Sucrose is the major translocated carbohydrate in plant tissue (Powell, 1990), and it is the most common carbon source used in anther culture, normally at levels of 2–4% (Reinert and Bajaj 1977). High sucrose levels (6–17%) are required in those species (e.g., Gramineae, Cruciferae) in which mature pollen is shed in the tricellular condition (Dunwell and Thurling 1985), whereas for those in which mature pollen is bicellular (e.g., Solanaceae) lower levels, such as 2–5%, are usually beneficial (Dunwell 2010). Sucrose is heat labile, and autoclaved media contain a mixture of sucrose, D-glucose and D-fructose (Powell, 1990). Maltose has also been added to anther culture medium of wheat, triticale, rye and rice at concentrations ranging from 60 to 90 g l^{-1} (Wedzony *et al.*, 2009).

Sucrose has been used as a major carbohydrate source in the induction medium. In rice, higher level of sucrose besides promoting the induction and growth of callus, is also useful in organogenesis (Chi-Chang-Chen, 1978) but the callus developed in a medium containing 9% sucrose differentiated into more of albino plants than those from low concentration of sucrose.

Reinert *et al.* (1977) suggested that sucrose level of 2 - 5% is good for rice anther culture. Sandhu *et al.* (1993) reported a high frequency regeneration of green plant in rice when lower concentration of sucrose was used (3% w/v) for callus inducton of anther.

Maltose has been reported to be a superior source of carbohydrate than sucrose for androgenesis in several species, including cereals (Last and Brettel 1990, Pande and Bhojwani 1999).

Various concentrations of sugar ranging from 1.5 to 12 per cent were tested. In general, a low concentration (3%) favoured androgenic initiation and callus induction, while a higher concentration (8%) inhibited callus formation (Rangaswamy *et al.,* 1992). The differentiation frequency of green plantlets was higher at 6 per cent than at 3 per cent sucrose (Clapham, 1973).

2.4.9 Exogenous hormones

2.4.9.1 Callus induction

A wide variety of growth stimulating hormones have been tried, singly and in numerous combinations. However, in rice, the response from these combinations was lower than 2, 4-D (2 mg l^{-1}) alone (Raina *et* al., 1987). Of-late, most researchers now employ several hormones as "combined initiation factors". In *Japonica* rice, Yang *et al.,* (1980) used a combination of 4 mg l^{-1} NAA, 1 mg l^{-1} 2, 4-D, and 1-3 mg l^{-1} kinetin. For indica rice, they used 2 mg l^{-1} 2, 4-D + 2 mg l^{-1} NAA + 3 mg l^{-1} kinetin. However, a combination keeping 2, 4-D at 1 mg l^{-1} and kinetin at 1-3 mg l^{-1} has been found especially suitable for subsequent regeneration.

Shahnewaz *et al.,* (2003) and Shahnewaz and Bari, (2004) observed that 2, 4-D (0.5 mg l^{-1}) in combination with the milder auxin NAA (2.5 mg l^{-1}), and kinetin (0.5 mg l^{-1}) induced callus from several indica varieties.

Roy and Mandal, (2005) used N6 (Chu *et al.,* 1975) medium with (2,4-D at 0.5, 1.0, 2.0 and 3.0 mg l^{-1}; NAA at 0.5, 1.0, 2.0 and 3.0 mg l^{-1}; IBA at 0.5, 1.0, 2.0 and 3.0 mg l^{-1}), organic adjuvants [(Yeast extract, at 200, 400 and 1000 mg l^{-1}; casein hydrolysate at 50, 250 and 500 mg l^{-1})

coconut water, at 5, 10 and 15%] and carbon sources (sucrose, maltose, dextrose, glucose, galactose and cane sugar) at 3 and 6% singly and 0.8% agar for callus induction in IR 72, Mansarovar, Pusa Basmati, Taraori Basmati, and Karnal local 95.

Hussain *et al.,* (2010) carried out investigations to standardize an efficient and effective protocol for callus induction, subsequent growth and regeneration in three Pakistani rice (*Oryza sativa* L.) varieties viz., GNY-53, Basmati-370 and JP-5. They used MS and N6 media for callus induction and they found that MS medium was better for callus induction as compared to N6 medium.

Gueye and Ndoye (2010) used N6 medium for five genotypes of *O. sativa* and three genotypes of *O. glaberrima*, for callus induction with 3 mg l^{-1} 2, 4-D, 1 mg l^{-1} NAA, 1 mg l^{-1} Kinetin and 0.8% w/v agar, and incubated at 27°C in the dark under 60% humidity to develop organogenic callus.

Thanh and Nguyen (2011) used N6 medium (Chu *et al.*, 1975), supplemented with 2 mg l^{-1} 2, 4-D, and 2 mg l^{-1} NAA for callus indction. Cultures were kept under dark condition with the temperature range of 25-28^{0}C.

2.4.9.2 Regeneration

In rice, all pollen calli do not regenerate (Rangaswamy *et al.*, 1992), and shoot differentiation from 50 per cent of the calli is considered very good. Best regeneration response was observed when calli ware transferred after 10-12 days of their emergence (about 2 mm size). Shoot induction could be achieved by transferring 2, 4-D induced pollen calli to IAA plus kinetin medium (Niizeki and Oono, 1968) or to hormone-free medium (Wang *et al.*, 1974). The relative ratio of IAA, NAA and kinetin also played a role in differentiation.

Chaleff and Stolarz (1982) obtained a high rate of regeneration using modified MS medium + NAA (2 mg l^{-1}) + kinetin (0.3 mg l^{-1}). Addition of coconut water (CW) in regeneration medium also improved the regeneration ability. The average induction frequency of green pollen plants, using all effective culture techniques, is more than 5 per cent for Japonica varieties and 1 per cent for indicas (Chu Chih-Ching, 1983).

Torrizo and Zapata (1986) demonstrated the effect of ABA on callus growth and plant regeneration in anther derived calli of rice. ABA at concentrations up to 4 x 10^{-6} M stimulated fresh weight increase in Taipei 309 and Fujisaka 5 while higher concentrations effected corresponding weight decreases in the three varieties tested. Relatively high ABA concentrations resulted in decreased callus size and production of more compact and whitish calli. ABA increased the frequency of calli producing green plants in Taipei 309 and the average green plant regeneration in all varieties tested.

Thuan *et al*, (2001) cultured anthers of F1 plants derived from four crosses of aromatic and improved rice cultivars in N6 and MS media supplemented with 2,4-D (0.5 mgL^{-1})+ NAA (1.0 mgL^{-1})+ BAP (0.5 mgL^{-1}) for callus induction. The anther-derived calli were subsequently subcultured in MS and N6 media supplemented with BAP (1.0 mg l^{-1}) and NAA (1.0 mg l^{-1}) for plant regeneration. Frequency of callus formation was better in N6 medium as compared to MS medium (11.9% and 7.95%, respectively). Anther-derived calli from the cross of Khao Hom Suphanburi and DS15 exhibited the highest regeneration (7.57% with 37 green plants) in N6 medium. Green plants could be regenerated in eight times higher in N6 medium than in MS medium.

Gioi and Tuan, (2002) carried out anthers culture studies in F1 plants derived from four crosses of new plant type and improved rice cultivars. Anthers were cultured in N6, LS and MS media supplemented

with 2,4-D (0.5 mg l^{-1}) + NAA (2.0 mg l^{-1}) for callus induction. The calli were subcultured in N6 medium supplemented with NAA (0.5 m l^{-1}) + BAP (2 mg l^{-1}) for plant regeneration. Frequency of callus formation was the best in N6 medium (3.53%) as compared to MS (2.63%) or LS medium (2.37%). Anther-derived calli from the cross of IR64 x IR68530 showed the highest plant regeneration (1.12% of inoculated anthers) response and no green plant was obtained in the cross IR64 x IR70441.

Haploid plantlets derived from embryogenic callus were found to be sterile, have short stems, narrow leaves, numerous tillers, small panicles and small spikelets when transplanted to the paddy field (Zepata and Arias 2003).

Roy and Mandal (2005) used MS medium supplemented with 1.0 mg l^{-1} BAP, 1.0 mg l^{-1} Kinetin, 0.5 mg l^{-1} NAA, 3% (w/v) sucrose and 0.8% (w/v) agar with a pH of 5.8, for regeneration.

Nadali et al. (2009) used MS5 modified culture medium (Murashige & Skoog 1962), with 1.0 mg l^{-1} (NAA) and 1.0 mg l^{-1} kinetin for regeneration.

Gueye and Ndoyr (2010) used MS medium with 1.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, incubated in a culture room at 27°C with a 14/10 hr photoperiod. Plantlets of 2 - 3 cm height obtained after 4 – 6 weeks culture were cultivated in MS medium without hormones for root development in five genotypes of *O. sativa* and three genotypes of *O. glaberrima*.

Thanh and Nguyen (2011) used MS medium with combination 1.0 mg l^{-1} BA, 2.0 mg l^{-1} Kinetin and 3% sucrose. Cultures were kept under 16 hrs daily illumination with 80-Watt fluorescent bulb.

2.4.10 Supplements

Lentini *et al.* (1995) reported that incorporation of $AgNO_3$ (10 mg l^{-1}) in the callus induction medium not only promoted anther response in indica rice but also the regeneration of green plants from the induced callus.

Reinert and Bajaj, (1977); Powell, (1990); and Achar, (2002) have reported supplementation of madia with glutamine, casein, proline, biotin, inositol, coconut water, silver nitrate (ethylene antagonist) and polyvinylpyrolidone for better response of anther culture.

Dewi *et al.* (2004) reported that putrescine is more efficient than spermidin and spermin in increasing callus induction and plant regeneration in anther culture of rice model Taipei-309.

Dewi *et al.* (2009) studied the plant regeneration of five indica rice genotypes, for use in breeding for tolerance to Al toxicity through anther culture using a basal medium containing putrescine.

Anther culture media are generally solidified by adding agar, but the beneficial effect of other solidifying agents, such as starch (potato, wheat, corn or barley starch), gelrite, agarose and ficoll, has been reported. Liquid, semisolid and two-phase systems in which anthers are floated on liquid medium overlying an agar-solidified medium have been tested with different results (Dunwell, 2010).

2.4.11 Albinism

During the regeneration of cereal microspore derived plants, both green and albino (chlorophyll- deficient) individuals are produced. Albinism is a major problem encountered in cereal anther and microspore culture. Many factors have been found to affect the degree of

albinism, such as the genotype and physiological state of the donor plants (Knudsen *et al.*, 1989).

The frequency of albino plants seems to be genotype dependent (Yang, 1988). Wang *et al.,* (1978) observed the presence of proplastids in leaf cells of albinos and Sun *et al.,* (1993) reported the absence of ribosomes and fraction I protein. No definite relationship could be established between the albinos and media components (Chu, 1975).

In rice, use of high sucrose concentration (9 to 12%) (Mercy and Zapata, 1986), high level of 2, 4-D or NAA (10 mg l^{-1}), (Chen, 1983) higher pre-treatment temperatures of panicles at 35°C for 3-5 days (Qu and Chen, 1986), higher incubation temperature of more than 25°C (Song *et al.,* 1978), and the physical environment of the donor plant (Huang *et* a*l.*, 1983) seem to increase the frequency of albinos. On the other hand, low pre-treatment temperature and 6 times higher strength of iron salts in N6 medium (Guo, 1983) seem to increase the frequency of green plants.

Sun *et al.* (1979) reported that the basic cause of albinism in rice is impairment of DNA (probably due to presence of chemicals added to the media) in plastids or nuclei or in both of them.

Shih-Wei and Zhi-Hong (1991) have reported that the frequency of albinism depended on the varieties or hybrids used, anther pre treatment temperature and the culture medium constituents.

Mohiuddin *et al.* (2011) reported albino plant regeneration from anther culture of Hobigonj Boro (Hbj B) IV and Hbj B VI, two local varieties of aromatic indica rice from Bangladesh. Three crucial factors were identified for the albino shoot primordia to change into green plantlets in culture; components of M10 induction medium, callus size (range 0.2–0.4 cm long) and height of shoot primordia (range 2–3 mm).

Immediate transfer of shoot primordia (2–3 mm) from M10 medium to regeneration medium followed by continuous incubation under fluorescent light (100-lux, 25±1°C) triggered albino shoot primordia to turn green in 2–3 days.

MATERIALS AND METHODS

III. MATERIALS AND METHODS

The present investigations on "**ANTHER CULTURE STUDIES IN RICE**" were carried out at the Plant Tissue Culture Laboratory, Department of Plant Biotechnology, University of Agricultural Sciences, GKVK Campus, Bangalore-560065 during the years 2011-12. The material and methods used for the study are described in this chapter.

3.1 Plant material

Rice varieties Azucena and Budda were used as the source of explants. Their specific characteristics are presented in Table 2. These varieties were grown in the field till the time of flowering. Recommended fertilizers and plant protection measures were adopted to raise healthy plants.

3.2 Media preparation

Stock solutions (macro, micro, Fe-EDTA and vitamins) were prepared initially by dissolving the analytical grade chemicals in required quantities in volumetric flasks using double distilled water. Iron-EDTA stocks were prepared and heated for a few minutes until it turned golden yellow and was then stored in a brown bottle in refrigerator for further use. Composition of the N6 medium used for callus induction and MS basal medium used for regeneration is given in Table 3 and Table 5 respectively.

3.2.2 Preparation of N6 and MS medium from stock solutions

The nutrients required for the N6 medium and MS medium were pipetted out in required quantities from the stock solutions (macronutrients, micronutrients, Fe-EDTA and vitamins) along with the required amount of sucrose 50g l^{-1} for callus induction in N6 medium

and 30g l^{-1} for regeneration in MS medium. Myo-inositol was weighed and added afresh each time to MS medium. After that specified final volume was made up with double distilled water than adding growth regulators. The pH was adjusted to 5.8 using 0.1N NaOH and 0.1N HCl. The required quantity of Agar (8 g l^{-1}) was added and the mixture was then heated for dissolving the agar. Then the medium was transferred to conical flasks making sure that each flask was filled with not more than half its capacity to ensure proper autoclaving. The flasks were plugged with tight cotton plugs and autoclaved at 121^0 C temperature and 15 lbs. pressure for 15 min.

After autoclaving, the media along with other materials (culture containers, para film etc.) were shifted to the laminar air flow hood, which was sterilized by UV for 15 min, before carrying out the experiment. After switching off the UV, the airflow and illumination was switched on. The floor of the cabinet and hands were swabbed with 70% alcohol to ensure total sterility. The media was poured into the petri plates/test tubes before it cooled down near the flames of Bunsen burner. Then, the media was allowed to solidify.

Table 2 : Characteristics of the rice varieties selected for the study

Parameters	**Azucena**	**Budda**
Type of subsp.	Japonica	Indica
Duration (days)	144.17	143
Yield(tonnes/ha)	4.75	2.70
Days to 50% flowering	104	82
Other features	Aromatic, high Fe, Zn content	Deep root system, Drought tolerant

3.3 Anther culture

3.3.1 Standardization of culture media for callus induction

3.3.1.1 Selection of explants

3.3.1.1.1 Stage of panicle harvest

Panicles were harvested at the early flowering stage, when young panicles were still enclosed within the leaf sheath. Panicles with a maximum distance of 7–13 cm between the subtending leaf and the flag leaf were selected.

3. 3.1.1.2 Cold pre-treatment

Panicles were collected between 8.00 and 9.00 am and washed with water and sprayed with 70 percent ethanol. These panicles were sealed in a polyethylene bag and were wrapped in aluminum foil. Cold pre-treatment was given by placing them in refrigerator at 5°C for 8 days.

3.3.1.1.3 Pollen growth stage

Spikelets were selected from three parts (top, middle and basal) of each panicle and anthers stained with acetocarmine and observed under a light microscope to identify the pollen development stage.

3.3.2 Sterilization and inoculation of the explant

Necessary precautions were taken to avoid contamination. On the day of inoculation, selected panicles were taken out the refrigerator and sterilized with 70 percent alcohol for 20 seconds followed by 0.2% $HgCl_2$ for 10 minutes in laminar air flow chamber. After 10 minutes, the sterilant was drained off and the panicles were thoroughly rinsed with sterile distilled water for 3-4 times. After that, the anthers were isolated from spikelet avoiding any mechanical damage followed by inoculation on petridish (60mm x 15mm) containing 10 ml of N6 solid basal medium.

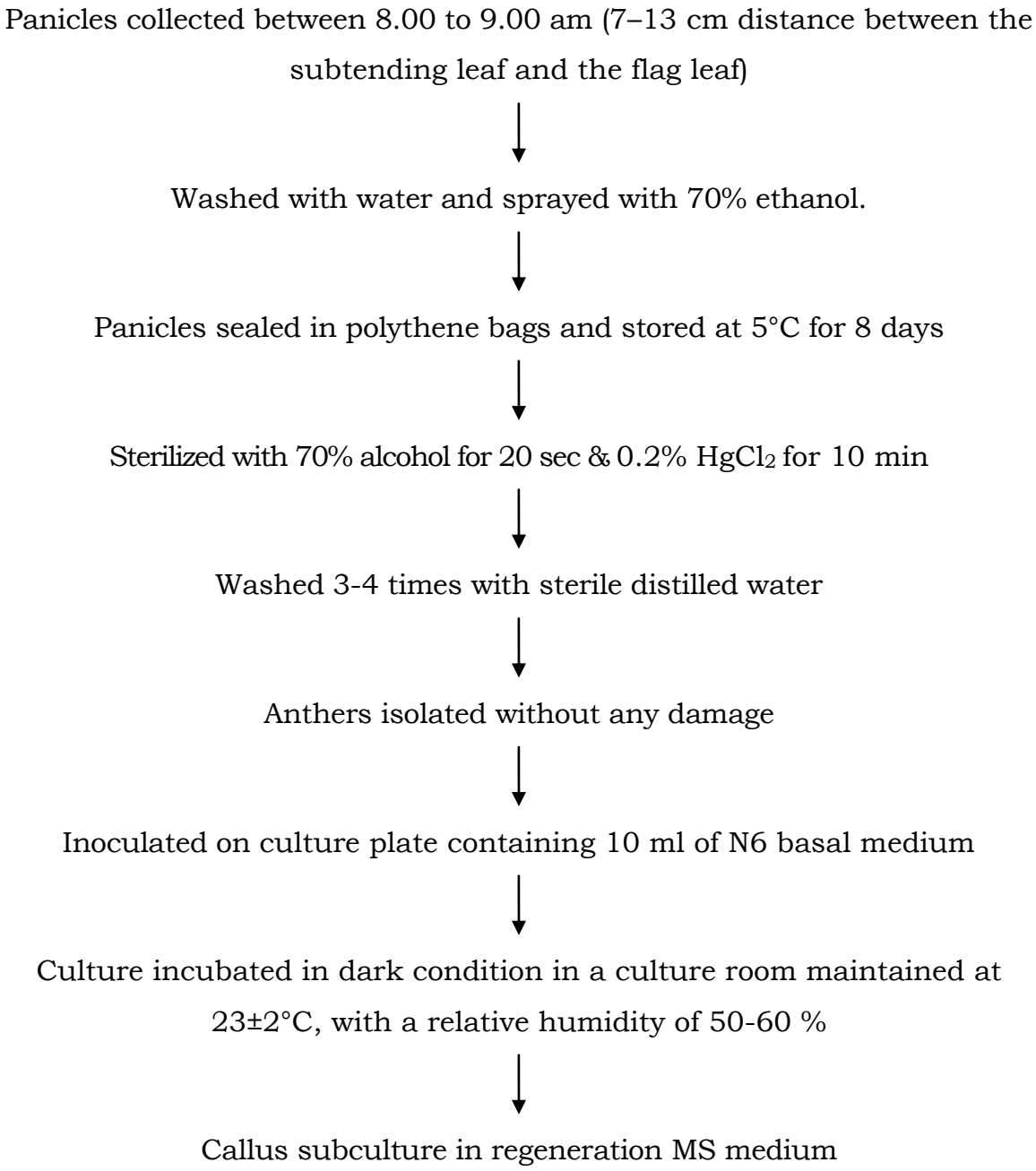


Figure 5 : Protocol for anther culture in rice

Table 3 : Composition of the stocks of N6 basal medium used for rice anther culture (Chu *et al.*, 1975)

Constituents	Medium composition (mg l^{-1})	Stock composition (g/250ml)
Stock 1 (10X)		
KNO_3	2830.000	7.075
(NH4)2SO_4	463.000	1.157
KH_2PO_4	400.000	1.0
MgSO4 . 7H_2O	166.000	0.4625
CaCl2. 2H20	166	0.415
Stock 2 (100X)		
KI	0.800	0.02
H_3BO_3	1.600	0.029
$MnSO_4.H_2O$	4.400	0.11
$ZnSO_4.7H_2O$	1.500	0.037
$CuSO_4.7H_2O$	0.025	0.000625
$CoCl_2.6H_2O$	0.025	0.000625
Na_2MoO4.2H_2O	0.250	0.0062
$FeSO_4.7H_2O$	27.800	0.696
Na_2.EDTA.2H_2O	37.500	0.93
Stock 3 (1000X)		
Thiamine HCl	1.000	0.125
Pyridoxine HCl	0.500	0.125
Nicotinic acid	0.500	0.25
The following constituents were freshly weighed and added seperately at the time of preparing the medium.		
Sucrose 5.0%		
Agar Agar 0.8%		
pH 5.8		

3.3.3 Treatments for callus induction

Treatment for callus induction was done using N6 as a basal medium with different concentrations of 2, 4-D, NAA and Kinetin (Table 4).

Table 4 : Treatments for callus induction from rice anthers

Treatments	2,4-D (mg l^{-1})	NAA (mg l^{-1})	Kinetin (mg l^{-1})
T_0	0.0	0.0	0.0
T_1	0.5	0.0	0.0
T_2	0.5	1.0	0.5
T_3	0.5	1.0	1.0
T_4	0.5	2.0	0.5
T_5	0.5	2.0	1.0
T_6	1.0	0.0	0.0
T_7	1.0	1.0	0.5
T_8	1.0	1.0	1.0
T_9	1.0	2.0	0.5
T_{10}	1.0	2.0	1.0
T_{11}	1.5	0.0	0.0
T_{12}	1.5	1.0	0.5
T_{13}	1.5	1.0	1.0
T_{14}	1.5	2.0	0.5
T_{15}	1.5	2.0	1.0
T_{16}	2.0	0.0	0.0
T_{17}	2.0	1.0	0.5
T_{18}	2.0	1.0	1.0
T_{19}	2.0	2.0	0.5
T_{20}	2.0	2.0	1.0

Basal medium- N6 media + 5% Sucrose + 8% Agar

3.3.4 Culture conditions for callus induction

All the cultures were incubated in dark condition in a culture room maintained at 23±2°C, with a relative humidity of 50-60%.

3.3.5 Observations

The cultures were observed frequently and the contaminated plates were removed. After 7 to 8 weeks of culture the following observations were recorded.

1. Number of anthers inoculated
2. Number of anthers producing callus
3. Weeks taken for callusing
4. Colour of the callus
5. Friability of the callus: Compact or loose
6. Callus growth
7. Callus induction frequency (%)

$$\text{Callus induction frequency (\%)} = \frac{\text{No. of anthers producing callus}}{\text{No. of anthers plated}} \times 100$$

3.3.6 Shoot Regeneration

After 50-60 days, anthers showing positive responses were transferred into test tubes (15 cm x 2.5 cm) containing 15ml of MS shoot regeneration medium.

Table 5 : Composition of Murashige and Skoog (1962) basal medium

Components	Concentration of salts in medium (mg l^{-1})	Salt Concentration in Stock solution (g l^{-1})
Macronutrients stock (10X):		
NH_4NO_3	1650	16.5
KNO_3	1900	19.0
$MgSO_4.7H_2O$	370	3.7
KH_2PO_4	170	1.7
$CaCl_2.2H_2O$	440	4.4
Iron stock (100X):		
$Na_2.EDTA.2H_2O$	37.25	3.72
$FeSO_4.7H_2O$	27.85	2.78
Micronutrients (100X):		
H_3BO_3	6.2	0.62
$MnSO_4.H_2O$	22.3	2.23
$ZnSO_4.7H_2O$	8.6	0.86
$CuSO_4.5H_2O$	0.025	0.0025
KI	0.83	0.083
$CoCl_2.6H_2O$	0.025	0.0025
$Na_2MoO4.2H_2O$	0.25	0.025
Vitamin stock (1000X):		
Glycine	2.0	2.0
Thiamine HCl	0.1	0.1
Pyridoxine HCl	0.5	0.5
Nicotinic acid	0.5	0.5
Myo-Inositol (100 mg l^{-1}) (Prepared freshly during media preparation)		
Sucrose (30 g l^{-1}) (Adjust the pH to 5.6-5.8)		
Agar (8.0 g l^{-1})		

3.3.6.1 Treatments for regeneration

The shoot regeneration treatments included MS basal media with different concentrations of NAA, BAP, Kinetin and Adenine sulphate (80mg l^{-1}). List of treatments to induce regeneration from callus are given in Table 6.

3.3.6.2. Culture conditions for regeneration

The cultures for regeneration were incubated in a culture room maintained at 23±2°C, with a relative humidity of 50-60% and 16-hour photoperiod at a photon flux density of 3000 lux from white cool fluorescent tubes.

3.3.6.3 Observations

All the cultures were observed frequently and the contaminated test tubes were removed. After 30 days of culture the following observations were recorded.

1. Number of callus transferred
2. Number of calli producing shoots
3. Number of regenerated plantlets
4. Colour of regenerated plantlets
5. Regenerated frequency (%)

$$\text{Regenerated frequency (\%)} = \frac{\text{No. of regenerated plantlets}}{\text{No. of calli plated for regeneration}} \times 100$$

Table 6: Treatments for regeneration from rice callus

Treatments	NAA (mg l^{-1})	BAP (mg l^{-1})	Kinetin(mg l^{-1})
T_0	0.0	0.0	0.0
T_1	0.0	0.0	0.5
T_2	0.0	0.0	1.0
T_3	0.0	0.0	2.0
T_4	0.0	0.5	0.0
T_5	0.0	1.0	0.0
T_6	0.0	2.0	0.0
T_7	0.0	0.5	0.5
T_8	0.0	0.5	1.0
T_9	0.0	0.5	2.0
T_{10}	0.0	1.0	0.5
T_{11}	0.0	1.0	1.0
T_{12}	0.0	1.0	2.0
T_{13}	0.0	2.0	0.5
T_{14}	0.0	2.0	1.0
T_{15}	0.0	2.0	2.0
T_{16}	0.5	0.5	0.5
T_{17}	0.5	0.5	1.0
T_{18}	0.5	0.5	2.0
T_{19}	0.5	1.0	0.5
T_{20}	0.5	1.0	1.0
T_{21}	0.5	1.0	2.0
T_{22}	0.5	2.0	0.5
T_{23}	0.5	2.0	1.0
T_{24}	0.5	2.0	2.0

Basal medium- MS media + 3% Sucrose + 8% Agar

Culture conditions- Cultures for regeneration were incubated in a culture room maintained at 23±2°C, with a relative humidity of 50-60% and 16-hour photoperiod at a photon flux density of 3000 lux from white cool fluorescent tubes.

All the experiments were conducted at the plant tissue culture laboratory, under uniform condition of temperature, humidity and light. For each treatment in the experiment, three replications were maintained. Statistical analysis was not done, because most of the treatments were not responding for callus induction and regeneration.

RESULTS

IV. EXPERIMENTAL RESULTS

The present investigation was carried out on anther culture in rice. Studies were carried out for standardizing culture media for callus induction in rice cv. Azucens and Budda. Standardization of callus induction and regeneration from callus for these cultivars was studied using various treatments of different growth hormones in N6 (Chu) basal medium. Regeneration of anther derived callus was carried out on MS basal medium. The results obtained from the above study are presented in this chapter.

4.1 Standardization of culture media for androgenic callus induction

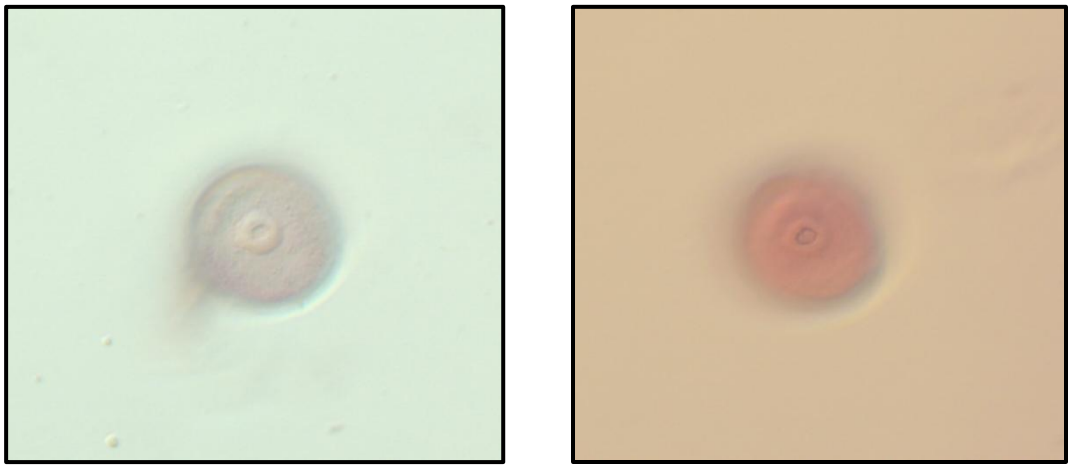
4.1.1 Stage of panicle harvest

Collection of panicles was done, with a maximum distance between the subtending leaf and the flag leaf, of 11cm for Azucena and 8 cm for Budda (Plate 1). These panicles were collected and kept in refrigerator for cold pretreatment at 5°C for 8 days. In order to identify the stage of pollen, anthers of Azucena and Budda were stained with acetocarmine and observed under light microscope. The observed pollens were uni-nucleate (Plate 2).

4.1.2 Androgenic callus induction in rice cv. Azucena with different concentrations of 2, 4-D, NAA and Kinetin.

The study was carried out to find out the effect of different concentrations of growth hormones on callus induction in rice cultivar Azucena and Budda. Anthers were used as explant for callus induction. The anthers were placed on N6 medium containing different concentrations of growth hormones, 2, 4-D, NAA and Kinetin for callus induction (Plate 3). They were incubated in dark for 7 to 8 weeks.

Plate 1 : Stage of panicle harvest in Azucena and Budda varieties. Distance between flag leaf and subtending leaf 11 cm in Azucena and 8 cm in Budda



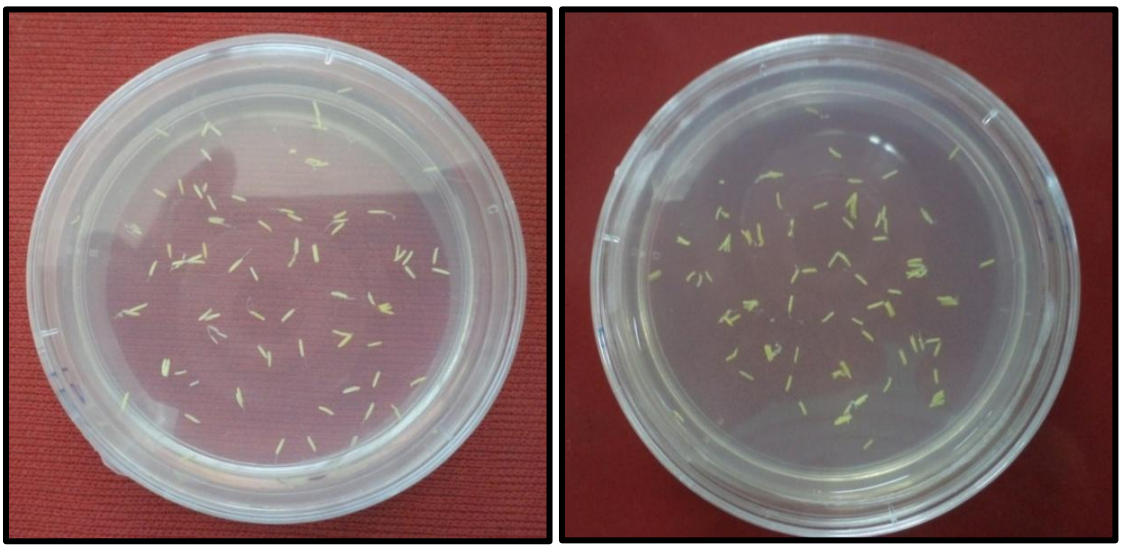
A **B**

Plate 2 : Pollen development stage observed under light microscope at 400x (Mid uninucleate) in Azucena (A) and Budda (B)

Cultured anthers turned black or brown in both the varieties after two weeks of culture. Callusing was not observed in Budda variety. First indication of callusing was swelling of the anther wall. Later callus appeared from the cut ends (Plate 4). It took 7-8 weeks for callus induction. Percent callus induction was variable and ranged from 0.66% T_4 (0.5 mg l^{-1} 2, 4-D, 2.0 mg l^{-1} NAA, 0.5 mg l^{-1} Kinetin) to 6.66% T_9 (1.0 mg l^{-1} 2, 4-D, 2.0 mg l^{-1} NAA, 0.5 mg l^{-1} Kinetin) in Azucena among 20 treatments. This result showed that only Azucena responded to callus induction in tried N6 medium with combination of growth regulators. Time taken for callus induction, percent callus induction and growth of callus were recorded and the data obtained is presented in Table 7.

4.1.2.2 Calli characteristics

Observations on colour of callus and type of callus were recorded (Table 8)**.** The colour of the callus was white in all the treatments except T_{12} (1.5 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin). Calli were seen to be compact in all the treatments. Per cent callus induction ranged from 0.66% in T_4 (0.5 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin) to 6.66% in T_9 (1.0 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin) on N6 medium, while in several treatments there were no response. The treatment T_3 (0.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin) showed maximum growth of callus production (Plate 5). Highest percent callus induction 6.66% in T_9 (1.0 mg l^{-1} 2,4-D, 2.0 mg l^{-1} NAA, and 0.5 mg l^{-1} Kinetin) (Plate 6) followed by 4.0% in T_3 (0.5 mg l^{-1} 2, 4-D, 1.0 mg l^{-1} NAA, and 1.0 mg l^{-1} Kinetin) was observed in Azucena variety.



A **B**

Plate 3 : Anthers inoculated on N6 medium, A-Azucena, B-Budda

Plate 4 : Induction of anther derived callus in Azucena rice variety (8 weeks after culture)

Table 7 : Effect of different concentrations of growth hormones on anther callus induction in rice variety Azucena

Treatment	No. of anthers inoculated*	Time taken for callus induction (Weeks)	No. of anthers Responded*	Callus Growth	% Callus induction
T_0	50	-	-	-	0.0
T_1	50	-	-	-	0.0
T_2	50	-	-	-	0.0
T_3	33.3	8	2	++++	4.0
T_4	50	11	0.33	+	0.66
T_5	33.3	7	0.33	+	0.66
T_6	33.3	-	-	-	0.0
T_7	33.3	-	-	-	0.0
T_8	33.3	-	-	-	0.0
T_9	50	6	3.33	+++	6.66
T_{10}	50	11	0.33	+	0.66
T_{11}	16.6	-	-	-	0.0
T_{12}	50	5	1.33	++	2.66
T_{13}	50	-	-	-	0.0
T_{14}	50	-	-	-	0.0
T_{15}	50	-	-	-	0.0
T_{16}	33.3	-	-	-	0.0
T_{17}	33.3	5	0.33	+	0.66
T_{18}	33.3	-	-	-	0.0
T_{19}	16.6	7	0.66	+	0.66
T_{20}	33.3	-	-	-	0.0

Basal medium- N6 media + 5% Sucrose + 8% Agar

Culture condition- Cultures for callus induction were incubated in dark condition in a culture room maintained at 23±2°C, with a relative humidity of 50-60%.

Legend:

Total number of anthers = 50 in each treatment

*	Average of 3 replication
-	No callus
+	Poor callus growth
++	Moderate growth
+++	Good callus
++++	Very good growth

T_0 = Control

T_1 = 0.5 mg l^{-1} 2, 4-D

T_2 = 0.5 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin.

T_3 = 0.5 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin.

T_4 = 0.5 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin.

T_5 = 0.5 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin.

T_6 = 1.0 mg l^{-1} 2, 4-D

T_7 = 1.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin.

T_8 = 1.0 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin.

T_9 = 1.0 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin.

T_{10} = 1.0 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin.

T_{11} = 1.5 mg l^{-1} 2, 4-D

T_{12} = 1.5 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin.

T_{13} = 1.5 mg l^{-1} 2, 4-D + 1.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin.

T_{14} = 1.5 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin.

T_{15} = 1.5 mg l^{-1} 2, 4-D + 2.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin.

T_{16} = 2.0 mg l^{-1} 2, 4-D

T_{17} = 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin.

T_{18} = 2.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin.

T_{19} = 2.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} NAA +0.5 mg l^{-1} Kin.

T_{20} = 2.0 mg l^{-1} 2,4-D + 2.0 mg l^{-1} NAA +1.0 mg l^{-1} Kin.

Note – These treatments were also used for callus induction in the variety Budda, but callusing was not found.

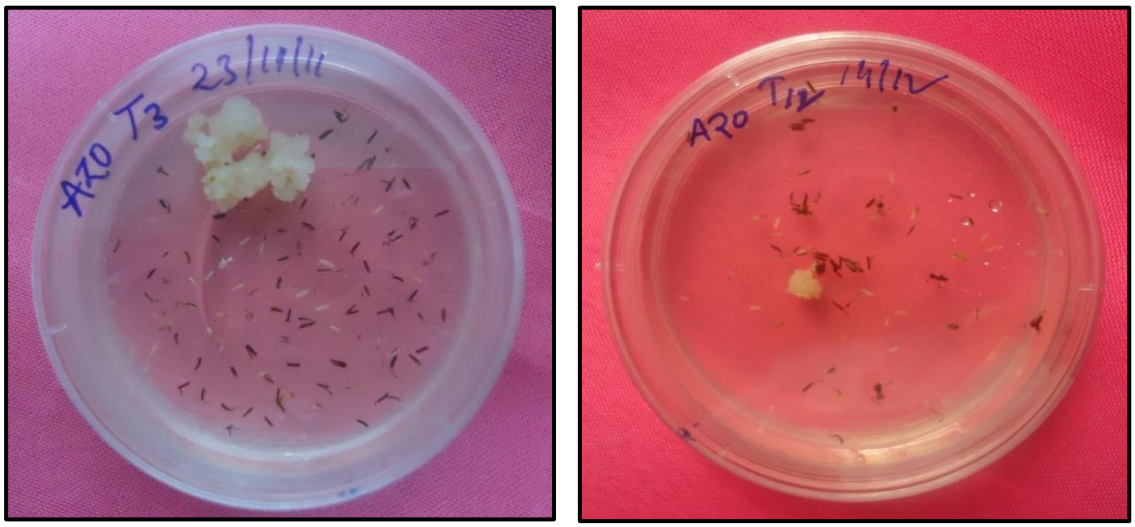


Plate 5 : Anther derived callus in Azucena rice variety after 10 weeks of culture

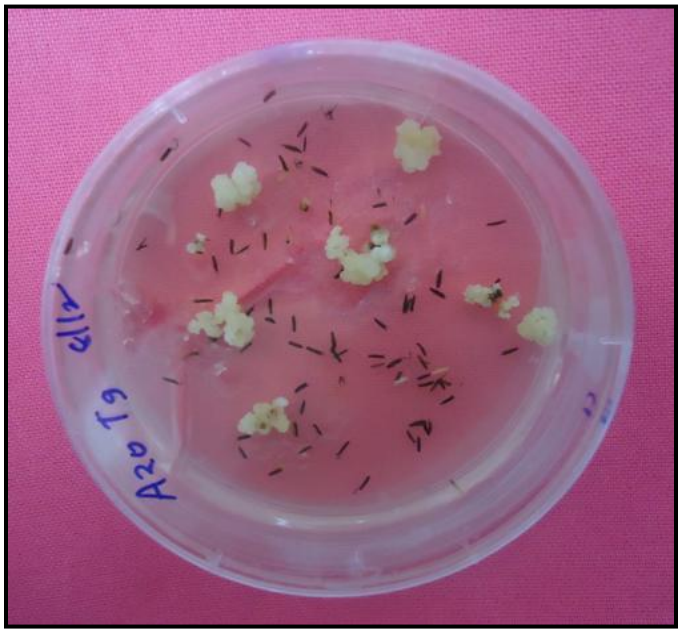


Plate 6 : Callus induction in T_9 (1.0 mg l^{-1} 2, 4-D, 2.0 mg l^{-1} NAA and 0.5 mg l^{-1} Kinetin) after 10 weeks of culture in Azucena variety

Table 8 : Physical characteristics of the callus of Azucena

Treatments	Colour of callus	Type of callus
T_0	-	-
T_1	-	-
T_2	-	-
T_3	White	Compact
T_4	White	Compact
T_5	White	Compact
T_6	-	-
T_7	-	-
T_8	-	-
T_9	White	Compact
T_{10}	White	Compact
T_{11}	-	-
T_{12}	pale yellow	Compact
T_{13}	-	-
T_{14}	-	-
T_{15}	-	-
T_{16}	-	-
T_{17}	White	Compact
T_{18}	-	-
T_{19}	White	Compact
T_{20}	-	-

Note: Treatments are same as Table 7.

4.2 Regeneration from anther derived calli

Calli derived from anthers of the variety Azucena were transferred to MS medium supplemented with various concentrations and combinations of NAA, BAP, kinetin, and adenine sulphate (Plate 7). In regeneration medium, most of the calli did not respond and died. After 15 days of culture, the callus turned green in colour in treatment T_7 (0.5 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin and 80 mg l^{-1} adenine sulphate) (Plate 8). Out of 24 treatments, T_3 (2.0 mg l^{-1} Kinetin and 80 mg l^{-1} adenine sulphate), T_5 (1.0 mg l^{-1} BAP and 80 mg l^{-1} adenine sulphate) T_6 (2.0 mg l^{-1} BAP and 80 mg l^{-1} adenine sulphate) and T_7 (0.5 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin and 80 mg l^{-1} adenine sulphate) responded positively for shoot regeneration (Table 9).

The treatment T_7 (0.5 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin and 80 mg l^{-1} adenine sulphate) showed shoot and root formation after 2 months of culture (Plate 9). Highest regeneration frequency of (0.33%) was observed in regeneration media containing 0.5 mg l^{-1} BAP, 0.5 mg l^{-1} Kinetin and 80 mg l^{-1} adenine sulphate in Azucena variety. The other treatments showed increase in callus size, but did not respond to shoot regeneration. The mean frequency of shoot regeneration for all treatments was 1.84%. All the shoots induced from anther derived calli were albinos in Azucena. However, shoots did not survive after one week of response in T_3, T_5 and T_6. Root formation was only observed in the regeneration medium without any growth regulator after 15 days of callus transfere (Plate 10).

Table 9 : Regeneration from anther derived callus in rice variety Azucena

Sl. No.	NAA (mg l^{-1})	BAP (mg l^{-1})	Kinetin (mg l^{-1})	Response	Time taken for Regeneration (days)
T_0	0.0	0.0	0.0	Root	15
T_1	0.0	0.0	0.5	-	-
T_2	0.0	0.0	1.0	-	-
T_3	0.0	0.0	2.0	Shoot	12
T_4	0.0	0.5	0.0	-	-
T_5	0.0	1.0	0.0	Shoot	15
T_6	0.0	2.0	0.0	Shoot	12
T_7	0.0	0.5	0.5	Shoot and Root	15
T_8	0.0	0.5	1.0	-	-
T_9	0.0	0.5	2.0	-	-
T_{10}	0.0	1.0	0.5	-	-
T_{11}	0.0	1.0	1.0	-	-
T_{12}	0.0	1.0	2.0	-	-
T_{13}	0.0	2.0	0.5	-	-
T_{14}	0.0	2.0	1.0	-	-
T_{15}	0.0	2.0	2.0	-	-
T_{16}	0.5	0.5	0.5	-	-
T_{17}	0.5	0.5	1.0	-	-
T_{18}	0.5	0.5	2.0	-	-
T_{19}	0.5	1.0	0.5	-	-
T_{20}	0.5	1.0	1.0	-	-
T_{21}	0.5	1.0	2.0	-	-
T_{22}	0.5	2.0	0.5	-	-
T_{23}	0.5	2.0	1.0	-	-
T_{24}	0.5	2.0	2.0	-	-

Basal medium- MS media + 3% Sucrose + 8% Agar

Culture conditions- Cultures for regeneration were incubated in a culture room maintained at 23±2°C, with a relative humidity of 50-60% and 16-hour photoperiod at a photon flux density of 3000 lux from white cool fluorescent tubes.

Legend: T = Treatment - = No response

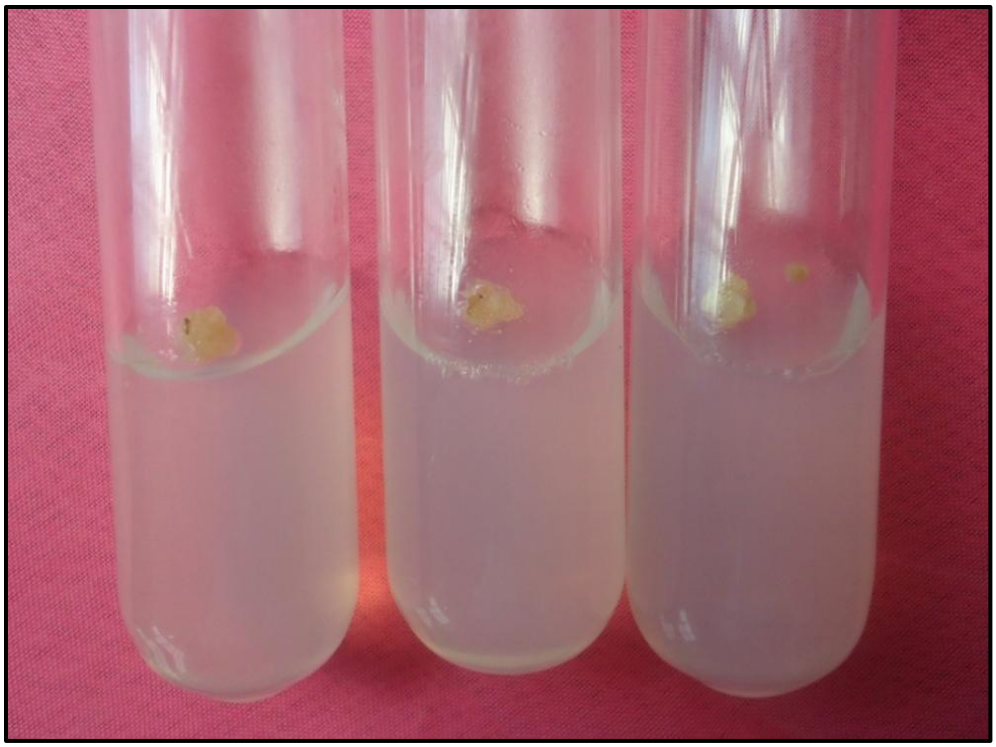


Plate 7 : Anther derived callus transferred to regeneration medium

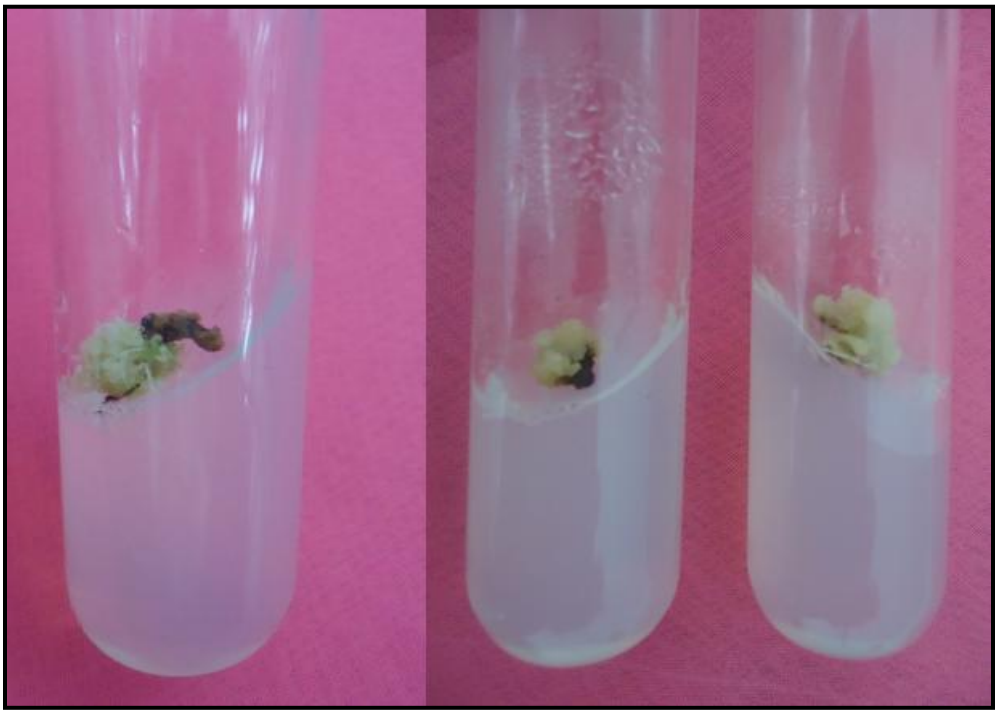


Plate 8 : Green callus produced in regeneration medium in Azucena after 15 days of culture

Plate 9 : Shoot and root formation in T_7 (0.5 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin and 80 mg l^{-1} adenine sulphate) from anther derived callus of Azucena after 2 months of culture (on regeneration medium)

Plate 10 : Root formation from anther derived callus of Azucena after 1 months of culture in T_0 (Control) regeneration medium

DISCUSSION

V. DISCUSSION

Plant breeding programs are highly complemented by tissue culture and genetic engineering techniques. In fact, the potential of tissue culture to generate *de novo* variability was realized as early as 1978 by Oono. Unfortunately, tissue culture techniques are often genotype specific and hence need extensive efforts in optimizing the technical details before venturing into the possible application of cell culture and genetic manipulation procedures.

Plant tissue culture techniques are recognized as useful instruments in crop improvement. Among these techniques, *in vitro* anther culture stands out as an increasingly powerful tool when integrated into breeding programs (Hu and Zeng, 1984). This technique allows the acceleration of plant breeding by providing homozygous doubled haploids within a comparatively short period of time (Nurhidayah *et al.,* 1996). Anther culture has been known to produce a high proportion of haploids (for chromosome doubling) or spontaneous homozygous dihaploids that can be further utilized in breeding process (Smykalova *et al.,* 2009).

Similar findings have been revealed by Rao *et al.* (2000) who reported that anther culture technique could be successfully utilized for the production of haploids which evoked considerable interest among plant breeders and genetists. The major advantage of this technique was the recovery of homozygous diploids for breeding and establishment of haploid cell cultures for mutational studies and genetic manipulation experiments. Extensive studies conducted on anther culture of rice has led to the identification of critical factors for successful development of haploids.

Various studies on rice anther culture have been reported by several workers (Gupta and Borthakur, 1987; Nadali *et al.*, 2009; Afza *et al.,* 2000; Islam *et al.*, 2004; Gueye and Ndoye, 2010; Thanh and Nguyen, 2011). The present investigation was conducted to standardize culture media for callus induction and regeneration from anther derived callus for rice cv. Azucena and Budda. The results of the investigation are discussed below.

5.1 Standardization of culture media for callus induction

5.1.1 Panicle harvest stage

Panicles were harvested at the early flowering stage, when young panicles were still enclosed within the leaf sheath. Panicles with a distance of 11 cm between subtending leaf and the flag leaf for Azucena and 8 cm for Budda were selected because at this position pollen was uni-nucleate. Similar kind of observations were also depicted by different researchers related to anther culture of rice. They have pointed out that panicles should be excised when it is enclosed by the sheath (Gupta and Borthakur, 1987). Panicle with a distance between subtending leaf and the flag leaf of 3-8 cm (Silva and Ratnayake , 2009); 7–13 cm (Afza *et al.*, 2000); 5-10 cm (Gioi and Tuan, 2002); 5-9 cm (Nadali *et al.*, 2009); 7–10 cm (Suriyan *et al.*, 2009); 5-10 cm (Gueye and Ndoye, 2010); 4 to 8 cm (Abbasi *et al.*, 2011) and 5-10 cm (Thanh and Nguyen, 2011) have been used for successful callus induction in different rice varieties.

Mercy and Zapata (1986) studied the distance between the flag leaf and the subtending leaf, as well as the late uninucleate and early binucleate pollen stage which has been used as markers for callus induction although with inconsistent success. Oono (1975), Chen and Lin (1976), Chen (1977) and Cornejo-Mart and Primo-Millo (1981) concluded that anthers at the tetrad stage showed no response at all, whereas, early

uninucleate pollen responded poorly while the mid-to late-uninucleate pollen responded the best.

In general, pollen grains at uni-nucleate to early bi-nucleate stages are considered to be optimum for the anther culture in many species (Sopory and Munshi, 1996; Goerecka *et al.,* 2005; Hu *et al.,* 1993). In most cases, the early-uninucleate to mid-uninucleate stage of microspores were found to be best suited for androgenic response (Datta, 1987; Datta and Potrykus, 1998; Sunderland and Dunwell, 1974; Jahne and Lorz, 1995).

Microspores can be induced to become embryogenic either *in planta* or in excised inflorescences or flower buds or in *in vitro* cultured anthers or isolated microspores (Touraev *et al.,* 1997). In all these cases, conversion to the sporophytic pathway can be induced by subjecting microspores to various stresses. Another important factor for successful conversion is the stage at which inflorescences, flower buds, anthers or microspores are excised. The salient results obtained are discussed belw.

5.1.2 Cold pre-treatment and dark incubation

The induction of microspores to sporophytic pathway instead of gametophytic pathway is strongly influenced by some kind of stress treatment of the anthers before culture. The response to chilling or heat treatment is also genotype dependent. However, a temperature shock has been reported to improve the androgenetic response in many plant species (Kiviharju and Pehu, 1998; Xie *et al.,* 1997). In graminaceous crops, it has been reported that cold-shock pre-treatment of young spikes was effective for anther culture of *Oryza sativa* (Zhou and Cheng, 1982). Zhou *et al.*, (1983) noticed variation in the requirement of cold shock pre-treatment among the *indica* and *japonica* rice genotypes. They also pointed out that when cold treatment duration exceeded a certain limit,

the induction frequency decreased substantially. In the present study cold pretreatment of 5°C for 8 days and was given for the selected panicles. The cultures were incubated in dark for 7-8 weeks for callus induction.

According to Sunderland *et al.* (1984), cold pretreatment promoted senescence of the anther wall, destroying the close association between tapetum and pollen. This might disturb the programmed microsporogenesis and allow embryogenesis to be initiated. Nitsch (1974) reported that cold shock triggers pollen to undergo asymmetric mitotic division which is a pre-requisite for callus induction. The combination of cold pretreatment and dark incubation had synergistic effect on callus induction frequency (Badigannavar, 1995).

5.1.3 Androgenesis induction medium

In the present study, after effective surface sterilization of pretreated panicles using 0.2% $HgCl_2$ for 10 min, sterilized anthers of the two varities were cultured *in vitro* on N6 (Chu *et al.*, 1975) basal medium with growth regulators (2,4-D, NAA, Kinetin) and 5% sucrose. Usage of N6 medium for culture of anthers had been reported previously by Reiffers and Adelson (1990); Afza *et al.*, (2000); Thuan *et al.*, (2001); Islam *et al.*, (2004); Gueye and Ndoye (2010) and Thanh and Nguyen (2011). An elevated sucrose concentration (4-6%) is mostly used for the preparation of the induction media for anther cultures (Nadali *et al.*, 2009; Thanh and Nguyen, 2011). In the present study, rice anthers showed a positive response on the N6 medium with a sucrose concentration of 50 g l^{-1} as reported by Islam *et al.* (2004). Several earlier reports (Chu *et al.*, 1975; Chen *et al.*, 1986 and Rangaswamy *et al.*, 1992) had indicated that N6 medium was the best for callus induction from anthers of rice. Thus N6 medium was selected in the present study.

5.1.4 Effect of growth regulators on callus induction

The kind and concentration of growth regulators are known to play an important role in androgenic response. Differences in androgenic response of anthers for callus induction were found among the treatments. In the present study callus induction was observed only in Azucena and no callus was produced in Budda. This indicates that genotype specific requirement of growth regulators is required to get a positive response. In this study, cultures were incubated in the dark for 7 to 8 weeks. Initial signs were that the anthers turned black or brown in colour. Later swelling of anther wall was noticed followed by appearance of callus at the cut ends. This observation is in accordance with the report of Gupta and Borthakar (1987). Percent callus induction due to growth regulators treatments were variable and ranged from 0.00% to 6.66% in Azucena and 0.00% in Budda. This result showed that callus induction response was observed only in Azucena.

Genotypic variation in androgenic response as observed in the present study in *japonica* rice and *indica* rice had been demonstrated earlier by many authors (Mandal and Bonyopadhyay, 1997; Gosal *et al.*, 1997; Raina and Zapata, 1997; Chen *et al.*, 1991). In rice, it has been demonstrated that *japonica* genotypes produce more androgenic callus and plants than *indica* genotypes (Zapata *et al.*, 1990; Yamagishi *et al.*, 1998).

In the present study, highest percent callus induction was 6.66% in T_9 (1.0 mg l^{-1} 2, 4-D, 2.0 mg l^{-1} NAA, and 0.5 mg l^{-1} Kinetin) followed by 4.0% in T_3 (0.5 mg l^{-1} 2, 4-D, 1.0 mg l^{-1} NAA, and 1.0 mg l^{-1} Kinetin) in N6 medium, while in several treatments there were no response. These results are consistent with the findings of Gueye and Ndoye (2010) as they observed 4.24% callus induction in IKP (*japonica*) variety on N6

medium supplemented with 3 mg l^{-1} 2, 4-D and 1 mg l^{-1} NAA and 1 mg l^{-1} Kinetin.

It was observed that the calli were compact in nature in all responded treatments and white/ pale yellow in colour (Table 8). Ranaweera (1998) obtained similar results in callus of rice genotypes.

Anther cultures of Budda (*Indica*) variety did not produce callus. Similar results were found by Ramkrishnan *et al.* (2005) in ADS16 (*Indica*); Silva and Ratnayake (2009) in Kurulu Thuda (*Indica*) and Shahnewaz and Bari (2004) in BRRI Dhan 29 (*Indica*). They also got similar results on N6 medium supplemented with 2, 4-D, NAA and Kinetin. Miah *et al.* (1985) reported that anther culture response varied from 41 % for a *japonica* cultivar to 0 % for an *indica* cultivar. The technique has been used successfully to produce homozygous breeding lines in *japonica* rice (Brar and Kush, 2006). However, the potential for *indica* rice anther culture is yet to be fully exploited due to various constraints that include a recalcitrant genetic background in the *indica* varieties (He *et al.,* 2006). A combination of auxin and cytokinin for callus induction was also used by many others (Afza *et al.*, 2000; Shahnewaz *et al.*, 2003; Islam *et al.*, 2004; Gueye and Ndoye 2010; Thanh and Ngnyen, 2011).

5.3 Regeneration from anther derived callus

The ability to regenerate plants from cultured cells or tissue at high frequencies is crucial for the successful application of tissue culture technology to crop improvement. This is one of the major areas of concern in rice. Many factors such as medium, growth regulators, culture environment, explant and genotype of donor are known to influence regeneration of plants.

The anther derived callus was transferred to MS basal medium along with various concentrations of NAA, BAP and kinetin. Reiffers and Adelson (1990); Afza *et al.* (2000); Thuan *et al.* (2001); Islam *et al.* (2004); Gueye and Ndoye (2010) and Thanh and Nguyen (2011) have also used MS medium with different combination of auxin and cytokinin.

In the present study, shoot and root formation from callus occurred on MS basal medium supplemented with 0.5 mg l^{-1} BAP, 0.5 mg l^{-1} kinetin and 80 mg l^{-1} adenine sulphate. These results indicate that BAP and kinetin are necessary for shoot induction. These results were consistent with the findings of Gueye and Ndoye (2010) who observed 5.75% regeneration frequency from callus in IKP (*japonica*) variety on MS medium supplemented with 0.5 mg l^{-1} NAA and 1.0 mg l^{-1} BAP. In the absence of any growth regulator in the media only root formation occurred in regeneration medium, which might be due to presence of endogenous auxin.

5.3.1 Albinos

Albinism is a serious problem in cereal anther culture (Bishnoi *et al.*, 2000). In the present study all the shoots regenerated from callus were albinos. They did not survive after one week of shoot induction, but one plant was pale yellowish in treatment T_7 (0.5 mg l^{-1} BAP, 0.5 mg l^{-1} kinetin and 80 mg l^{-1} adenine sulphate). Many factors have been found to affect the degree of albinism, such as the genotype and physiological state of the donor plants (Knudsen *et al.*, 1989). As per the earlier reports, the mean induction frequency of green plant regeneration, using all effective culture techniques, was about five per cent for *japonica* and less than one per cent for *indica* (Rangaswamy *et al.*, 1992). Sun *et al.* (1979) reported that the basic cause of albinism in rice is impairment of DNA might occur either in nuclear genome or cytoplasmic genome. This

might be due to presence of chemicals such as high sucrose concentration, high level of 2, 4-D or NAA added to the medium.

Conclusion

The present investigation indicates that response of anther culture was better in Azucena (subsp. japonica) variety of rice compared to the rice variety namely, Budda (subsp. indica). It was also evident from the study that the androgenic response was highly genotypic specific.

Future line of work

1. Albinism can be reduced by shortening culture period or shorter pre-cold treatment.
2. Confirmation of haploid plants by chromosome counting approach or ploidy level determination by flow cytometry.

SUMMARY

VI. SUMMARY

Among the different plant tissue culture techniques, *in vitro* anther culture has been recognized as one of the powerful tools for crop improvement when integrated into breeding programs, by producing haploids or homozygous dihaploids. Rice (*Oryza sativa* L., 2n=24) is one of the most versatile and important cereal crops. The present study was designed to standardize the culture media for callus induction and regeneration from anther derived callus in two rice varieties, Azucena (subsp. japonica) and Budda (subsp. indica).

Anthers from the panicle with a maximum distance of 11cm and 8 cm length between the subtending leaf and the flag leaf in Azucena and Budda respectively were selected for the study. At this length anthers contained uninucleate pollen grains.

Before inoculation of the anthers, a cold pretreatment of 5°C for 8 days was given to the selected panicles in order to induce microspores in them to follow the sporophytic pathway instead of gametophytic pathway.

Anthers with uninucleate pollens were used for callus induction. The cultures for callus induction were incubated in dark condition in a culture room maintained at 23±2°C, with a relative humidity of 50-60%. The frequency of callus induction was genotype dependent. The anthers of the Azucena variety, responded to callus induction but in the variety Budda no callusing was found when inoculated on N6 medium with different concentrations of 2,4- D, NAA and Kinetin.

Anthers of Azucena responded with maximum percent callus induction (6.66%) on solid N6 medium containing 5% sucrose, 1.0 mg l^{-1} 2, 4-D, 2.0 mg l^{-1} NAA and 0.5, mg l^{-1} Kinetin.

Anther derived calli of the variety Azucena were transferred to MS basal medium with various concentrations of NAA, BAP and Kinetin for regeneration. The cultures for regeneration were incubated in a culture room maintained at 23±2°C, with a relative humidity of 50-60% and 16-hour photoperiod at a photon flux density of 3000 lux from white cool fluorescent tubes. Highest regeneration frequency of (0.33%) was observed in regeneration media containing 3% sucrose, 0.5 mg l^{-1} BAP, 0.5 mg l^{-1} Kinetin and 80 mg l^{-1} adenine sulphate in Azucena variety. All the shoots regenerated were albinos. In the absence of any growth regulator in the media only root formation occurred in regeneration medium, which might be due to presence of endogenous auxin.

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APPENDICES

APPENDIX - I

List of abbreviations used in the text and their expansions.

2, 4-D = 2, 4- Dichlorophenoxy acetic acid

ABA = Abscisic acid

B5 = Gamborg medium

BA = 6- Benzyl adenine

BAP = 6- Benzyl amino purine

CW = Coconut water

GA = Gibberellic acid

$HgCl_2$ = Marcuric chloride

IAA = Indole -3- acetic acid

IBA = Indole -3- butyric acid

Kin = Kinetin

LS = Linsmaier and Skoog

MS = Murashige and Skoog

mg l^{-1} = Milligram per litre

NAA = 1- Naphthalene acetic acid

N6 = Chu medium

APPENDIX - II

NUTRITIONAL VALUE OF RICE

Rice provides energy of 1527 kJ (365 kcal),

Nutritional value per 100 g (3.5 oz)

Carbohydrates	80 g
Sugars	0.12 g
Dietary fiber	1.3 g
Fat	0.66 g
Protein	7.13 g
Water	11.61 g
Thiamine (Vit. B_1)	0.0701 mg (6%)
Riboflavin (Vit. B_2)	0.0149 mg (1%)
Niacin (Vit. B_3)	1.62 mg (11%)
Pantothenic acid (B_5)	1.014 mg (20%)
Vitamin B_6	0.164 mg (13%)
Calcium	28 mg (3%)
Iron	0.80 mg (6%)
Magnesium	25 mg (7%)
Manganese	1.088 mg (52%)
Phosphorus	115 mg (16%)
Potassium	115 mg (2%)
Zinc	1.09 mg (11%)

Source: USDA Nutrient database.