PROTOPLAST CULTURES OF *Oryza sativa* L. AND *Brachiaria decumbens*

By

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LIST OF ABBREVIATIONS

AA medium	Amino acid medium
ANOVA	Analysis of Variance
BAP	Benzyl amino purine
Cm	Centimetre
CMS	Cytoplasmic male sterile
CRBD	Complete Randomized Block Design
cv.	Cultivar
2,4-D	2,4-Dichlorophenoxyacetic acid
DNA	Deoxyribonucleic acid
F_1	First filial generation
Ft.	Foot/feet
g/L	Gram per litre
IBA	Indole butyric acid
IOA	Iodoacetamide
IRRI	International Rice Research Institute
KI	Potassium iodide
LS	Linsmaier and Skoog
M	Meter
Mb	Mega basepairs
mg/L	Milligram/Liter
mL	Millilitre
Mm	Millimeter
MS	Murashige and Skoog

NAA	1-Naphthalene acetic acid
PCR	Polymerase Chain Reaction
PEG	Polyethylenegycol
PGR	Plant growth regulator
RAPD	Random Amplification of Polymorphic DNA
RFLP	Restriction Fragment Length Polymorphism
Rpm	Rotation per minute
RSV	Rice stripe virus
µg	Microgram
°C	Degree Celcius
°F	Degree Farenheit
µL	Microlitre
%	Percentage

LIST OF PUBLICATIONS

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KULTUR PROTOPLAS *Oryza sativa* L. dan *Brachiaria decumdens*

ABSTRAK

Sistem kultur tisu bagi padi (*Oryza sativa* L.) cv. Fujisaka 5 dan IRAT 13, dan *Brachiaria decumbens* telah berjaya dibangunkan. Teknik pensterilan dua peringkat dengan menggunakan 70 % etanol dan 20 % Clorox® adalah memadai untuk memperolehi 83.3 % hingga 100 % biji benih aseptik bergantung kepada spesis dan kultivar padi yang digunakan. Peratusan percambahan biji benih yang tinggi iaitu sebanyak 97.7 % telah dicapai untuk *O. sativa* cv. Fujisaka 5. Manakala *O. sativa* cv. IRAT 13 dan *B. decumbens* menunjukkan peratusan percambahan yang rendah, iaitu, masing-masing 28.0 % dan 40.0 %. Kalus *O. sativa* L. cv. Fujisaka 5 pada permulaannya diaruh daripada 3 jenis eksplan, biji benih matang, akar dan daun. Eksplan akar dan biji benih matang *O. sativa* L. cv. Fujisaka 5 menunjukkan tindak balas yang baik bagi penghasilan kalus dari segi biojisim manakala tiada kalus dihasilkan daripada eksplan daun. Medium pepejal MS + 2.0 mg/L 2,4-D adalah mencukupi untuk merangsang pembentukan kalus *O.* sativa. Kalus embriogenik dan bukan embriogenik dihasilkan daripada eksplan benih matang. Walaubagaimanapun, kalus *O. sativa* L. cv. Fujisaka 5 yang diaruh daripada eksplan akar menunjukkan pembentukan akar rerambut halus pada permukaan kalus selepas beberapa kitar pengsubkulturan. Kalus yang dihasilkan tidak membeza, berwarna kuning keputihan dengan pertumbuhan akar serta pertumbuhan yang perlahan. Embriosomatik telah dikenalpasti pada medium MS yang mengandungi sama ada NAA atau BAP sahaja atau gabungan kedua-duanya, sungguhpun peratusan embrio yang bercambah adalah rendah dalam julat 0.1 - 0.8 % daripada berat basah. Medium MS yang mengandungi

1.0 mg/L NAA didapati berkesan untuk regenerasi *O. sativa* cv. Fujisaka 5. Embriosomatik *O. sativa* cv. Fujisaka 5 membentuk plantlet normal selepas disubkulturkan ke dalam medium MS yang tidak mengandungi pengawal atur pertumbuhan. Kultur ampaian sel *O. sativa* cv. Fujisaka 5 telah dibangunkan daripada kalus berusia dua minggu dan menunjukkan keputusan yang terbaik dalam medium cecair Amino asid + 2.0 mg/L 2,4-D + 0.2 mg/L kinetin (Medium AA). Teknik pengsubkulturan menggunakan pipet telah digunapakai untuk menyediakan sel ampaian yang halus. Pemilihan enzim adalah penting untuk pemencilan protoplas. Empat jenis kepekatan campuran enzim Cellulase Onozuka R10 dan Macerase Pectinase telah diuji. Protoplas dapat dipencilkan daripada kultur ampaiansel *O. sativa* cv. Fujisaka 5 dan IRAT 13, mesofil daun dan kalus digunakan untuk *B. decumbens* dengan menggunakan 1.0 % Cellulase Onozuka R10, 0.1 % Macerase pectinase, 0.1 % Polyvinylpyrrolidine-10, dan 5 mN MES yang dilarutkan dalam CPW 13M pada pH 5.8. Teknik inkubasi semalaman telah digunakan dan menghasilkan sel yang mencukupi untuk kultur protoplas. Walau bagaimanapun, kultur protoplas ini gagal menghasilkan mikro koloni.

PROTOPLAST CULTURES OF *Oryza sativa* L. AND *Brachiaria decumbens*

ABSTRACT

The tissue culture systems for the rice (*Oryza sativa* L.) cv. Fujisaka 5 and IRAT 13, and *Brachiaria decumbens* were established. Two stage surface sterilization techniques using 70 % ethanol and 20 % Clorox® was sufficient to obtain 83.3 % to 100 % aseptic seeds depending on species and rice cultivar used. A high seed germination percentage of 97.7 % was obtained for *O. sativa* cv. Fujisaka 5. While, *O. sativa* cv. IRAT 13 and *B. decumbens* showed low germination percentage of 28.0 % and 40.0 % respectively. Callus of *O. sativa* L. cv. Fujisaka 5 was initially induced from three explants, mature seeds, roots and leaves. The root explants and the mature seeds of *O. sativa* L. cv. Fujisaka 5 showed good response in callus production in term of biomass while no callus could be produced from the leaf explants MS solid medium + 2.0 mg/L 2,4-D was sufficient to induce formation of *O. sativa* callus. Embryogenic and non embryogenic calli were produced from the mature seed explants. However, the root derived callus of *O. sativa* L. cv. Fujisaka 5 produced fine root hairs on the callus surface after several subculture cycles. The calli produced were unorganized, yellowish white in colour with root formation and slow in growth. Somatic embryos were observed on MS medium containing either NAA or BAP alone or in combination, although the percentage of germinated embryos were low ranging from 0.1 – 0.8 % of total fresh weight. MS medium supplemented with 1.0 mg/L NAA was found to be effective for regeneration of *O. sativa* L. cv. Fujisaka 5. Somatic embryos of *O. sativa* L. cv. Fujisaka 5 regenerated into normal plantlets after subcultured into the PGR free MS medium. Cell suspension culture of *O. sativa* L. cv. Fujisaka 5 was established from two weeks old

callus and showed the best result in liquid Amino acid medium + 2.0 mg/L 2,4-D + 0.2 mg/L kinetin (AA medium). The pipetting technique of subculturing was used to prepare a fine cell suspension cultures. The selection of the enzyme solution is very important for isolation of protoplast. Four concentration of enzyme solution containing Cellulase Onozuka R10 and Macerase Pectinase were tested. Protoplasts were isolated from cell suspension culture of *O. sativa* cv. Fujisaka 5 and IRAT 13, while leave mesophyll and callus were used for *B. decumbens* using 1.0 % (w/v) Cellulase Onozuka R10, 0.1 % (w/v) Macerase pectinase, 0.1 % (w/v) Polyvinylpyrrolidine-10, and 5 mN MES dissolved in CPW 13M at pH 5.8. The overnight incubation method was used and enable to produce sufficient cells for protoplast culture. However, protoplast culture using liquid culture method and agarose bead techniques failed to form microcolonies.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Rice is the staple food for the more than 50% of the world's population (Liu et al., 1999). About 90 % of rice are produced in Asia. Rice is produced in a wide range of locations from the wettest areas in the world to the driest deserts (Maclean et al., 2002). Rice provides 21 % of a global human per capita energy and 15 % of per capita protein requirements. Rice also provides minerals, vitamins and fiber, although all constituents except carbohydrates are reduced by milling. The demand for rice is now a major concern and many factors have contributed to the rice crisis such as imbalance between demand and production, extreme weather, pest problem and diseases (IRRI, 2008).

The rice research has benefited the rice farmers all over the world. Rice research is a key role that develops new technologies for all farmers in meeting this need and contributing to global efforts directed at poverty alleviation. Research has provided 75 % of the rice varieties now grown (Aliyu et al., 2013). In 2013, IRRI and their partners have released 44 new and improved rice varieties planted in 11 countries (IRRI, 2014). It also increases yield potential from 4 to more than 10 tonnes per hectare crop. FAO has set target to boost rice production and livelihood of farmers by transformed rice sector to be more productive, efficient and environmentally sustainable by 2030 (IRRI, 2014). Research has been a major factor in more than doubling world rice production from 260 to 600 million tonnes over the past 40 years. Research that have been carried out has enable the farmers to produce produced rice plants that grow faster, with two or even three crops per year,

resistance to various pests and diseases, require less fertilizer, or able to thrive in saline water and grow with enhanced levels of micronutrient (Maclean et al., 2002).

Rice production has been increased in recent years because of different factors such as improvement of agronomic practices, release of new varieties resistant to disease and pests, development of varieties tolerance to biotic and abiotic stress, increased irrigation and the use of fertilizers (Khush, 2003). However, human population in developing countries has increased faster than the rice production, thus increased production of this staple food crop is still a major objective of plant breeders. Since suitable land for high yielding varieties are very limited, the increase of rice production must come from increased in yield per hectare, cropping intensity and utilization of marginal land. In such cases, varieties must be developed which have higher yield potential, superior grain quality, resistance to disease and pests and tolerance to abiotic stress. The objectives of plant breeders are to improve rice varieties through either conventional or non-conventional breeding methods (Lee et al., 1999). For this purpose, plant biotechnology is utilized in conjunction with conventional breeding methods in order to improve the rice varieties.

Plant biotechnology has generated considerable interest for further improvement of crop plants. The integration of this technology into conventional breeding has enabled breeders to achieve several targets that were inconceivable in the past within a short period of time (Guimaraes, 2009). Plant biotechnology is seen as additional tool for the plant breeder and not as substitute for traditional breeding techniques where by various conventional and biotechnology approaches are being employed to develop new varieties (Khush, 2005). Transformation and somatic hybridization have recently been applied to transfer of gene in rice improvement programmes. Genetic variability is the key to any further genetic improvement. The

plant cell culture techniques have increased the genetic variability even more rapidly from somatic cells by tissue or protoplast culture (Lee et al., 1999). Introduction of the genes of interest from its wild species relative is one way to increase the genetic variability of the crop plants (Liu et al., 1999).

Brachiaria decumbens Stapf., a tropical and perennial savanna grass native to Africa possess numerous traits valuable for rice breeding such as disease and insect resistance, cold and flooding tolerance and high grain quality (Shelton, 2000). But, *O. sativa* L. and *B. decumbens* is sexually incompatible thus preventing the cross-transfer of genes between them. In cases like this, the genetic transformation method can be used if the genes have been cloned (Liu et al., 1999). Otherwise, somatic hybridization through protoplast fusion is an alternative approach to enable the integration of agronomically important genes from donor to recipient. For both of these genetic manipulation approaches, tissue culture is required as a tool to allow plant regeneration from the manipulated cells.

For such genetic manipulation studies, it is necessary that the cell or protoplast systems regenerate to the complete plant. In this regards, Abdullah et al. (1986) were the first to describe the reproducible regeneration of fertile rice plants from the cell suspension-derived protoplasts of Japonica rice. Since then, more efficient plant regenerations have been reported from protoplasts of Japonica and Indica rice. Before the regenerated plants could be achieved, all the process starting from callus initiation, establishment of cell suspension culture, isolation of the protoplast and protoplast culture have to be carried out.

1.2 Research Objectives

The present study was carried out with the following objectives:

1. To establish the aseptic cultures of rice (*O. sativa*) cv. Fujisaka 5 and IRAT 13 and *Brachiaria decumbens*.
2. To establish the callus cultures of rice (*O. sativa*) cv. Fujisaka 5 and IRAT 13, and *Brachiaria decumbens.*
3. To establish the cell suspension cultures for rice (*O. sativa*) cv. Fujisaka 5 and IRAT 13, and *Brachiaria decumbens.*
4. To isolate and prepare protoplasts from the cultured cells of rice (*O. sativa*) cv. Fujisaka 5 and IRAT 13, and *Brachiaria decumbens.*

CHAPTER 2

LITERATURE REVIEW

2.1 The rice crop (*Oryza sativa* L.)

Rice (*Oryza sativa* L.) is the primary food source for more than a third of the world's population. Rice cultivars are planted over 150 million hectares with production of about 600 million tonnes in the world (Gowda et al., 2003). More than 90 % of the world's rice is grown and consumed in Asia alone, where 60 % of the Earth's people live (Brar and Khush, 2006). Rice is the most important crop in developing countries. Asian countries dominated the rice production with 133 million hectare, while Latin America and African countries each produce only roughly 8 million hectare and developed countries including Japan produce only 5 million hectare (Evenson, 2004). Rice is produced in several different environments. The dominant production environments are irrigated and rainfed 'paddy' environment. Rice is also produced in 'upland' and 'deepwater' environment (Evenson and Gollin, 2002).

Recent advances in agriculture enhanced rice productivity to a higher level in order to meet world population demand. Superior rice cultivars such as IR32, IR 36, IR 40, IR 42, IR 64 and IR72 were developed by IRRI by conventional breeding method (Gowda et al., 2003). Different factors had attributed to increase rice production such as, improved agronomic practices, release of new varieties resistant to disease and pests, development biotic and abiotic stress tolerance varieties, increased irrigation and the use of fertilizers (Khush, 2001). Population growth in many developing countries is continuing at more than 2 % annually. So, further increases in agricultural output of this staple crop are essential for global political

and social, and still the major objective of plant breeders (Tilman et al., 2002). For this purpose, innovative breeding methods and the emerging tools of molecular genome must supplement the conventional breeding methods (Gowda et al., 2003).

Plant biotechnology is seen as perhaps future hope to improve rice varieties with superior grains quality, increase genetics yield potential, enhanced resistance to pests and diseases, and resistance to several biotic and abiotic stresses such as drought, cold and nutrient deficiencies (Borlaug and Dowswell, 2003). The integration of plant biotechnology into conventional breeding has enabled breeders to achieve their target within a short period of time because biotechnology has the techniques to speed up the whole process (Snape, 2004). Transformation and somatic hybridization have been applied to gene transfer in rice improvement program. Somatic hybridization enables the transfer of alien genes material to plants parasexually, thereby overcoming sexual incompatibility between the crop species and unrelated species or organisms (Waara and Glimelius, 1995). Hence, transformation enables the integration of agronomically important genes. Tissue culture technology is required as a tool to allow plant recovery using the manipulated cells of both genetic manipulation approaches.

2.1.1 Economic importance of rice

Rice is of vital importance to the economy of developing countries and nutritionally, produces more calorific intake and carbohydrate per hectare than any other cereal crop. It produces 20 % of the world's dietary energy supplies, good source of vitamins 13 % of the protein for human consumptions, and its economic importance is second only to wheat (FAO, 2004). In terms of protein, analysis of over 4000 samples of rice at IRRI (International Rice Research Institute) showed a

protein range from 5.6 % to 18.2 % in the rice grain. The increasing world population would essentially need a continuous increase in food production (Davey et al., 2000). The world's human population is expected to increase to 8.0 billion by the 2020, and more than half of this population would be primary rice consumers. However, it is estimated that the world's annual rice production must increase to at least 700 million tonnes (equivalent annually to a 3 % increase) by 2025 (Papademetriou, 2000). In order to meet this demand, new strategies and approaches, clearly have to be incorporated into traditional rice breeding programmes.

World rice production in 2000 was about 600 million tonnes. At least 114 countries grow rice and more than 50 have an annual production of 100,000 tonnes or more. Asian farmers produce about 90 % of the total, with two countries, China and India, growing more than half of the total crop (Maclean et al., 2002). For most rice producing countries where annual productions exceed 1 million tonnes, rice is the staple food. In Bangladesh, Cambodia, Indonesia, Laos PDR, Myanmar, Thailand and Vietnam, rice provides 50 % – 80 % of the total calories consumed. Notable exceptions are Egypt, Nigeria, and Pakistan where rice contributes only 5 % – 10 % of per capita daily caloric intake (Maclean et al., 2002).

The typical Asian farmer plants rice primarily to meet family needs. Nevertheless, nearly half the crop goes to market; most of that is sold locally. Only 6 % – 7 % of world rice production is traded internationally. The major rice exporters are Thailand, the United States, Vietnam, Pakistan, India and China (Maclean et al., 2002). Developing countries account for 95 % of the total world rice production, 83 % of exports and 85 % of imports, with China and India accounting for more than 50 % (Emani et al. 2008).

Rice is a good source of thiamine, riboflavin and niacin. Brown rice is a good source of dietary fiber. The overall amino acid profile for rice seed shows high values for glutamic and aspartic acid, with lysine as the limiting acid. Rice is an integral part to the culinary traditions of many cultures with personal preferences regarding texture, taste, color and stickiness (FAO, 2004). Dry flaky rice is preferred in South Asia and the Middle East; moist sticky rice in Japan, Taiwan, Korea, Egypt, and Northern China; red rice and long-grained scented recipies such as sushi (Japan), paella, risotto, and pancit (Italy) (Emani et al., 2008).

2.1.2 The genus *Oryza*, classification, origin and morphology of cultivated rice.

Cultivated rice (*O. sativa* L) belongs to the family Poaceae (Gramineae), subfamily Bamboosoideae, and tribe Oryzeae. This tribe has 11 genera, of which genus *Oryza* is the only one with cultivated species (Brar and Khush, 2006). *Oryza* is modest-sized genus consisting of 20 well-recognized wild species and two advanced cultivars, *O. glaberrima* and *O. sativa* (Chang, 2002). *O. sativa* subspecies *indica* and *japonica* are largely grown and consumed in the world (Gowda et al., 2003).

Kingdom	: Plantae
Division	: Magnoliophyta
Class	: Liliopsida
Order	: Poales
Family	: Poaceae (Gramineae)
Subfamily	: Bamboosoideae
Tribe	: Oryzeae
Genus	: *Oryza*
Species	: *O. glaberrima* and *O. sativa*

Rice is a semiaquatic, monocarpic annual grass plant, its height varying from 0.6 to 1.8 m (2 – 6 ft.) tall. The plants tiller developed multiple shoots, depending on the variety, spacing, and soil fertility. The grass has long, slender leaves 50 – 100 cm long and 2 – 2.5 cm broad. The small wind-pollinated flowers are produced on a branched arching to pendulous inflorescence 30 – 50 cm long. The inflorescence is open panicle. Flowers are distinct in having six anthers as opposed to the commonly seen three anthers in other grasses. Spikelets have a single floret, lemma, and palea enclosing a grain (caryopsis) 5 – 12 mm long and 2 – 3 mm thick that can be yellow, red, brown or black. The lemmas may be awn-less, partly or fully awned. The rice kernel has four primary components: the hull or the husk, the seed coat or bran, the embryo or germ, and the endosperm (Emani et al., 2008).

Cultivated rice is generally considered a semi-aquatic annual grass, although in the tropics it can survive as a perennial which producing new tillers from nodes after harvest (rationing) (GRiSP, 2013). At maturity, the rice plant has a main stem and several tillers. Each productive tiller has a terminal flowering head or panicle. Plant height varies by varieties and environmental conditions, ranging from approximately 0.4 m to more than 5 m in some floating rice. The morphology of rice is divided into the vegetative phase (including germination, seedling and tillering stages) and the reproductive phase (including panicle initiation and heading stages) (Maclean et al., 2002).

The rice grain, commonly called a seed, consists of the true fruit or brown rice (caryopsis) and the hull, which encloses the brown rice. Brown rice consists mainly of the embryo and endosperm. The surface contains several thin layers of differentiated tissues that enclose the embryo and endosperm (Maclean et al., 2002).

Each stem of rice is made up of a series of nodes and internodes. The internodes vary in length depending on variety and environmental conditions, but generally increase from the lower to upper part of the stem. Each upper node bears a leaf and a bud, which can grow into a tiller. The tillering stage starts as soon as the seedling is self-supporting and generally finishes at panicle initiation. The major structures of the panicle are the base, axis, primary and secondary branches, pedicel, rudimentary glumes and spikelets (Maclean et al., 2002). The rice root system consists of two major types: crown root (including mat roots) and nodal roots (Maclean et al., 2002).

The growth duration of the rice plant is 3 - 6 months, depending on the variety and the environment under which it is grown. During this time, rice completes two distinct growth phases; vegetative and reproductive. The vegetative phase is subdivided into germination, early seedling growth and tillering. The reproductive is divided into the time before and after heading or panicle excertion. The time after heading is better known as the ripening period. Potential grain yield is primarily determined before heading. Ultimate yield, which is based on the amount of starch that fills the spikelets, is largely determined after heading (Mae, 1997).

The ideal climate for rice growth is 75 º F (24 º C). In non-industrialized nations, rice fields are prepared by ploughing (by cattle-drawn or a traitor), fertilizing (traditionally with dung or sewage) and smoothing (a process of dragging a log across the field). The process will reduce the percolation of water, nutrient losses and weed growth (Mather and Trinh Ton That, 1984). Seedlings are prepared in seedling beds, and after a month, are transplanted manually to the fields, which have been flooded by rain or river water. Dike-controlled canals or manual watering maintains

irrigation during the entire growth period. Before harvesting the crop, the fields are allowed to drain (Emani et al. 2008).

The small size of the *Oryza sativa* genome is reflected in its nuclear content of DNA (430 Mb), when compared to common wheat [13,240 Mb] (Chang, 2002). Nearly 450 genes has been identified in rice which affect the biotic and abiotic stresses, colouration of plant parts, morphological, physiological and biochemical traits (Gowda et al., 2003).

2.1.3 The uses of rice

Rice is the main item of the diet and it is frequently the basic ingredient of every meal. Rice is normally prepared by boiling or steaming. In Asia, bean curd, fish, vegetables, meat and spices are added depending on local availability and economic situation (Maclean et al., 2002). A small proportion of rice is consumed in the form of noodles, which serve as a bed for various, often highly spiced, specialties or as a bulk ingredient in soups. Glutinous rice plays an important role in some cultures. Glutinous rice is a staple food on Laos PDR and Northeast Thailand. In other cultures, it is prepared in a sweetened form for snacks, desserts or special food for religious or ceremonial occasions. In a few areas, glutinous rice is pounded and roasted to be eaten as a breakfast cereal (GRiSP., 2013). In Milan, Risotto is one of the most important and beloved traditional Italian dishes (Concetti, 2013).

Alcoholic beverages made from rice are found throughout the rice-producing world. The most common is a rice beer produced by boiling husked rice, inoculating the mix with a bit of yeast cake, and allowing the mixture to ferment for a short period (Maclean et al., 2002).The mash left at the bottom of the container is often prized. Among the Ifugao of the Philippines, the mash is frequently reserved for the

village priest. Among the Kachins of Myanmar, it is the first food offered to a recently captured and hungry wild elephant. Kachins believed that the elephant will be loyal forever to the person who first provides such a meal (Maclean et al., 2002).Sake is widely consumed in Japan, as is wang-tsiu in China. These rice-based wine-like beverages are served warm and featured at ceremonial feasts (Maclean et al., 2002).

In some parts of the world, especially in North America and Europe, rice is developing a new market niche as a staple and as a gourmet food. This trend appears to be related to the arrival of large numbers of immigrants from Southeast Asia, who introduced aromatic rice to market where it was previously unknown. It has been adopted by a food quality-conscious public over the past several years (Maclean et al., 2002). Rice contains many compounds in the grains that promote shiny hair and good skin. Several countries are now making face washes, liquid shower soaps, and hair products from rice, including Japan, Korea, Philippines and Thailand (GRiSP, 2013). In much of Tanzania, rice is used for making bread; in the south, it is also used in ceremonies. In West Africa, rice bread, rice cake and rice porridge are used for ceremonies such as funerals and weddings. Some 'old' varieties (most likely *O. glaberrima*) are used in traditional religious rituals in West Africa, while certain parts of some varieties are used as medicines in the traditional treatment of illnesses (Maclean et al., 2002).

An extensive list of other ways of using rice is given by FAO:

- Milled rice is marketed precooked, canned, dried, and puffed for breakfast cereals as rice flour; extrusion-cooked foods; puddings and breads; cakes and crackers; noodles and rice paper; fermented foods and vinegars; rice starch; and syrups.

- Rice bran, which forms 5 % to 8% of the grain weight, is used as livestock feed, a pickling medium (a medium for growing mushrooms, and as a growing medium for some enzymes, as well as for flours, concentrates, oils and dietary fiber.
- Hulls and husks, about 20 % of grain weight, are used for fuel, bedding, and incubation material, and as a seedbed medium, as well as being sometimes incorporated in livestock feeds, concrete blocks, tiles, fiberboard, ceramics, cement, filters, charcoal briquettes, and cooking gas production.
- Rice straw, more or less equivalent in production weight to grain, it used as fuel for cooking, roofing material, livestock feed, fertilizer and a medium for growing mushrooms (GRiSP, 2013).

2.2 Gene transfer by conventional breeding methods

2.2.1 Objective of rice breeding

The target of rice breeding programme is development of high quality and high yielding rice cultivars. The core of plant breeding is the selection better cultivars, in terms of yield and grain quality, tolerance to environmental stress and resistance against pests (Breseghello and Coelho, 2013).The rice breeding programme using hybridization was first initiated by Kano in 1904 in Japan (Grist, 1983). Rice breeding programmes extensively undertaken by the IRRI in the Philippines, were set up in the mid 1960's. An investment of USD 12 million in rice research has returned more than USD 70 million in benefits for rice farmers and national economics in Bangladesh, Indonesia, Vietnam and Philipines (IRRI, 2013). Rice breeding work has concentrated on developing high yielding potentials, better

nutritional quality, improved grain quality and resistance to biotic and abiotic stress with higher nutrient and water use efficiency (Singh et al., 2015).

Traditionally rice varieties encompass a huge range of potentially valuables genes (Hettel, 2014). The major agronomic traits include: lifecycle time, weed competitiveness, nitrogen (and other macronutrient) uptake and utilization, plant height, number of tillers and panicles, photoperiod sensitivity, flowering time, wide compatibility, male sterility and/or self incompatibility, fertility restoration, grain number, weight, size, shape, fragrance, composition and nutritional quality including starch, proteins, macro- and micronutrients (Emani et al. 2008). The genes linked to valuables traits can help breeders create new rice varieties that have improve yield potential, higher nutritional quality, ability to grow in problem soils and improve tolerance of pests, diseases and the stresses such as flood and drought (Hettel, 2014).

Major advances have been made in increasing rice productivity. World rice production more doubled from 257 million tonnes in 1966 to 599 million tonnes in 2000 (Brar and Khush, 2006). This was mainly achieved through the application of classical Mendelian genetics and conventional plant breeding. Mendel's laws of inheritance explained about the law of segregation whereby during gamete formation, the alleles from each gene segregate from each other so that gamete carries only one allele for each gene. After that, Mendel came out with law of independent assortment and later law of dominance (Strachan and Read, 1999). The current world population of 6.1 billion is expected to reach 8.0 billion by 2030, and rice production must increase by 50 % to meet the growing demand (Khush and Brar, 2003). Further, several biotic (diseases, insects) and abiotic (drought, salinity, iron and aluminum toxicity) stresses lower rice productivity. To overcome these constraints, there is an urgent need to develop rice varieties with higher yield

potential and durable resistance to diseases, insects and abiotic stresses (Brar and Khush, 2006).

2.2.2 Yield considerations

Increasing yield potential of rice has always been the priority in working to increase food security (Ebron, 2013). Although fertilizer tends to increase non-irrigated rice yields, the response is often irregular because of environmental stresses. As a consequence, fertilizer used is limited and yields are also low compared with irrigated rice, which has controlled water supplies and high inputs of fertilizer and pesticides. The yield potential of deepwater rice could be improved by introducing an appropriate plant type (Maclean et al., 2002).

To meet high yield required for rice production, plant breeders have developed nitrogen-responsive, semi-dwarf, high tillering varieties. This effort resulted in an increase in the rice yield from 2 - 3 tonnes/hectare to 8 - 10 tonnes/hectare in the tropics (Virmani et al., 1993). To sustain increasing rice production, rice breeders have concentrated their own efforts in producing high yielding varieties (Chang, 1984). In Africa, a new generation of high performing rice varieties has been launched with a yield advantage of 30 – 50 % (IRRI, 2013).

Another area for improvement of rice production is through the production of F_1 hybrids. This method has increased rice production by 20 % -30 % more than the best high yielding varieties (Virmani and Edwards, 1984; Yuan and Virmani, 1986) in China. Approximately 8.4 million hectares are planted with hybrid rice in China alone (Yuan and Mao, 1991).From the rice research programmes, the scientists have discovered a gene that enables rice plant to produce around 20 % more grain by increasing uptakes of phosphorus in the lowest value phosphorus-deficient land

(IRRI, 2012). The discovery allows them to grow more rice and adding global production and enhance their income. Germany is also supporting IRRI and other partners in the Hybrid Rice Development Consortium in improving genetic materials and related research on hybrid rice (Ferrer, 2013).

2.2.3 Disease and pest resistances

Rice field having a tremendous diversity of animals, plants, and microorganisms which is some of it are harmful to the rice crop and many of which are beneficial. Emphasis is placed on breeding rice varieties with resistance to insect pests and diseases and on minimizing the use of pesticides to promote natural biological control by beneficial insects, spiders and microorganisms (Maclean et al., 2002).

A rice pest is any organism that causes economic loss in rice production, including arthropods (insects and mites), pathogens (bacteria, fungi and viruses), weeds, mollusks (snails), and vertebrates (rodents and birds) (GRiSP, 2013). In a study by IRRI, it was found that the farmers lost on average of 37 % of the rice yield to pests and disease and these loses can range between 24 % and 41 % depending on the production situation (Sparks et al., 2012). The damage they do ranges from severing stems or killing tissue to competing with the crop for nutrients and sunlight (Maclean et al., 2002). For example, bacterial blight disease can damage as much as 60 – 70 % of the plant and can even result in crop failure, especially when disease strikes at the seedling stage (Reyes, 2013). Although pests and disease can be controlled by using chemicals such as pesticides and fungicides, these are expensive and also introduce danger to the environment and health. Thus, development of resistance plant to disease or pests is an important to preserve the related area. The

preferred route is to produce ideal plant type with integrated pest and disease resistance genes (Diehl and Bush, 1984). Plant diseases and pests are strongly influenced by changing weather patterns and these climate changes make it complex. However, the discovery of Pi9 gene is effective against blast populations tested so far (Mohapatra, 2013).

Screening techniques for evaluating germplasm have been carried out in many discipline related to the development of a source of resistance in rice and its use. The development of insect-resistant cultivar provides a substantial economic return to country investment. The traits in resistant donor plant will be identified and transferred to existing susceptible cultivars using conventional breeding methods (Smith, 2000). Jena and Khush (1990) successfully transferred brown planthopper (BPH) resistance from *O. Officinalis* into cultivated rice. An international cooperation among plant pathologist, plant breeders, entomologists, physiologists and agronomists in the breeding of IR36, thirteen rice cultivars from six different countries and a species of rice, *O. nivara*, were utilized (Innes, 1992). In year 2000, José Pons and the team have successfully obtained the transgenic plant via an *Agrobacterium tumefaciens* transformation system.

New biotechnology-based approaches are being applied to product new insect and disease resistant varieties. DNA marker-assisted selection has been used to increase the efficiency of breeding for pest resistance and to enable the “pyramiding” of multiples genes for resistance to a single insect or disease. Wide hybridization techniques enable resistance genes to be introduced from wild rice species. Genetic engineering is being employed to introduce resistance to stem borers and sheath blight (Maclean et al., 2002).

Besides the pests and diseases problems, rats problem have also decreased the rice yield production. Every year, Vietnam loses 10 % of the rice production. But, using ecologically based rodent management resulted in 93 % less rodent damage in rice area, a 10 – 14 % increase in rice yield, 20 % higher returns and 50 % less rodenticide use (IRRI, 2012).

2.2.4 Abiotic stress tolerance

Abiotic factors such as salinity, drought, cold, flooding severely limit the rice production. Since suitable land for high yielding varieties has been very limited, the increase of rice production must come from increases in yield per hectare, cropping intensity and utilization of marginal land (GRiSP, 2013). The climate change starts to have significant impacts on the conditions affecting lands in rice-producing regions (Barona-Edra, 2013). In such cases, varieties must be developed which have higher yield potential, superior grain quality, resistance to disease and pests and tolerance to abiotic stress. Swarna-Sub 1, a smart rice variety is made to especially thrive in environments affected by flooding, drought, cold temperatures and soils that are too salty and contain too much iron which lead to iron toxicity (IRRI, 2014). A scientist team from Japan and CIAT has discovered the DR01 genes that make the roots of the rice plants grow downward instead of outward. This allows the plants to reach the water deeper in the soil (Palmer, 2013). Plants with DR01 can continue to grow and produce grain even under extreme weather stress.

Instead of breeding the rice stress tolerance, a simple irrigation technique developed by IRRI can cut down water use by as much as 25 % in producing rice (IRRI, 2014).

2.2.5 Grain quality and nutritional value

Improving the nutritional value of rice is one of the main aims in the field of rice economics. The nutritional aspects of rice become an important consideration in the context of improvement, because it supplies most of the energy and protein for dependent population it (Bajaj, 1991). Grain size and shape, milling quality, amylase content, cooking quality and protein content of rice have been studied (Grist, 1983). The Grain Quality and Nutrition Center team at IRRI evaluates physical traits (chalkiness, head rice yield, milling potential and grain dimension) and several biochemical traits (amylase content, gelatinization temperature, gel consistency, viscosity, grain elongation and aroma) as a something interest and also important factor during milling process (Ebron, 2013).Rice provided 19 % of global human per capita energy and 13 % of per capita protein in 2009. Unmilled (brown) rice of 17,587 cultivars in the IRRI germplasm collection contains averages of 9.5 % protein. Rice also provides minerals, vitamins, and fiber, although all constituents except carbohydrates are reduced by milling. Milling removes roughly 80 % of the thiamine from brown rice. A precook rinsing or a boiling of milled rice results in additional loss of vitamins, especially B1 (GRiSP, 2013).

With the approach of genetic engineering to breed a crop, the invention of Golden Rice has give promises to help reducing the deaths and blindness because of insufficient vitamin A in poor communities around the world (Purugganan, 2013). Besides that, a team of researchers had designed a new strain (MucoRice-ARP1) contains antibody *arp1* which could potentially fight rotavirus that cause severe diarrhea (IRRI, 2013).

2.2.6 The limitation to conventional rice breeding methods

The efficiency of conventional rice breeding is low because of long term selection and backcrossing. Continuous selection of domesticated wild rice for the development of high yielding and well adapted varieties has led to genetic erosion which narrowing rice variability (Gowda et al., 2003). The success of any crop improvement programme depends on the extent of genetic variability in the base population and always available to the breeders (Khush and Virmani, 1985; Bajaj 1991). However, there is a lack of genetic variability in cultivated rice and, in many cases, variability in the cultivated species for important economic traits is limited (Khush, 1991). Moreover, the transfer of some useful traits from wild species to cultivated rice using sexual crossing are impossible because of sexual incompatibility.

2.3 Role of hybridization and gene introgression in rice improvement using biotechnology option

Somatic hybridization and cybridization have great potential in plant improvement. Somatic hybridization of plants by protoplast fusion is a technique that combines somatic cell from two different cultivars, species or genera in an effort to regenerate novel germplasm (Guo et al., 2004). Somatic hybridization also increased the genotypic variability in crops by transfer the resistance/tolerance to biotic and abiotic stress genes from wild species (Jelodar et al., 1999). While, cybridization is a technique to get fusion products that have one nuclear gene of one parent and cytoplamic genes of the other parent. Cybrids can be prepared by fusing a normal protoplasts with enucleated protoplast (Sathyanarayana, 2007). Recently, researchers

are studying the use of cybrids to transfer the resistance genes which is carried on the chloroplast genome.

The aim of wide crossing is to introduce useful alien genes from wild relatives of *O. sativa* into commercially useful improved varieties (Jena and Khush, 1990). It has rarely been used in rice improvement programmes in the past, mainly due to the problem of sexually incompatibility.

In many cases, appropriate genes are not available within the cultivated rice themselves and fortunately, wild species are an important reservoir of useful genes. In some cases, important genes have already been transferred into a few crop species through wide hybridization. For example, the transfer of a resistance gene to grassy stunt virus from a strain of *O. nivara* to *O. sativa* and transfer of the cytoplasm of *O. perennis* to *O. sativa* develop new cytoplamic male sterile (CMS) lines of rice for hybrid seed production (Khush et al., 1977; Lin and Yuan, 1978).

Cytoplamic male sterility is the most effective genetic tool for developing hybrid rice. Well-developed hybrid seed production practices give average yields of 2 tonnes/hectare of seed. Seed yields of 1 - 2 tonnes/hectare have been obtained by commercial seed growers in India, Vietnam and Philiphines (Maclean et al., 2002).

2.4 *Brachiaria decumbens*Stapf.

Brachiaria decumbens Stapf. is a tropical and perennial savanna grass native to Africa. This species has also found in other tropical country such as Central and South America, southern Asia and the Pacific region (Loch, 1977). It spread naturally by seed dispersal. Natural tetraploid (2n=36) populations are widespread, whereas diploid population are rare. The genera of *Brachiaria* constitute very important forage crops which need genetically improvement (Naumova et al., 1999).

There are 97 species of the genus *Brachiaria* distributed throughout the tropics especially in Africa (Renvoize et al., 1996). *B. decumbens* is a vigorous and trailing perennial grass with short, dark green leaves, rooting from the lower nodes of erect culms. These arise from a long prostrate, stoloniferous base to 30-45 cm high when vegetative and up to 1 m high when in flower. The inflorescence is a secund panicle of 2 – 4, more or less curved, racemes attached at right angles to the rhachis of the panicle and spreading horizontally. Spikelets have a male lower floret and a fertile hermaphrodite upper floret, and are closely contiguous and generally arranged in two ranks (Loch, 1977).

2.4.1 *Brachiaria decumbens* Stapf., source of disease and insect resistant and abiotic stress tolerance for rice

B. decumbens possess numerous traits valuable for rice breeding such as disease and insect resistance, cold and flooding tolerance and high grain quality. They grow in a wide range of habitats such as swamp, light forest shade, semi-desert and savannah (Renvoize et al., 1996) and adapted to infertile acid soil (Keller-Grain et al., 1996). Beside of that, *B. decumbens* can grow in the dry season as long as 7 months and rainfall as low as 1,300 mm.

2.5 Tissue culture of rice and protoplast technology

Plant tissue culture is the culturing of cells, tissues, organs or whole plant into a sterile culture bottle containing defined culture medium under controlled environmental conditions (Thorpe, 2007). Mohan (2001) and Shah et al. (2012) used plant tissue culture as a research tool for crop improvement, while Prathanturarug et al. (2012) and Ren et al. (2012) conserved the endangered and rare plant species. This technique has become an important and practical alternative in achieving

sustainable and stable agriculture as it reduces labour, time and cost (Garcia-Gonzales et al., 2010). Hence, plant cell cultures also important tools for the valuable secondary metabolites (Vanisree et al., 2004).

2.5.1 Callus induction of rice

Callus is basically a more or less non-organized tumour tissue which usually arises on wound of differentiated tissues and organs. This process is exploited in *in vitro* cultures where a tissue is wounded and the induced callus is further cultivated on solid culture medium with suitable ratio of plant growth regulators (Street, 1973). The first rice callus from stem nodes cultured on Heller's medium, was reported by Furuhashi and Yatazawa (1964). Since then, rice improvement programmes have been developed using rice cell and tissue culture. Rice callus can be produced easily from different explants such as those of roots, shoots, leaves, ovary, pollen and endosperm (Bajaj, 1990; Mustafa et al., 2011). It is possible with many different plant species to produce a callus and then grow this further on a new medium. Monocotyledons react differently when considering callus induction, generally being less to form callus tissue than dicotyledons. It is often only necessary to add auxin as the hormonal stimulus for callus induction such as 2,4-D or NAA (Mandal and Dutta Gupta, 2003; Rajesh et al., 2003). After induction, the callus is grown further on a new medium/fresh medium. The first subculture is usually carried out onto a solid medium or in some cases it is possible to begin directly with a liquid medium (Martin, 2003). If the callus has poor growth during the first subculture then it may be necessary to also subculture other part of the explants. The growth conditions (nutrient medium, physical growth factors) are usually similar to those for callus induction, or with lower auxin and cytokinin concentration (Pierik et al., 2012). If the

growth of the callus stagnates after subculturing then this indicates that the subculture medium is not suitable.

Rice callus induction and plant regeneration from different explants utilizes several media, such as AA2 (Muller and Grafe, 1978), MS (Murashige and Skoog, 1962), LS (Linsmaier and Skoog, 1965), N6 (Chu et al., 1975) and R2 (Ohira et al., 1973). Composition of the culture media influences success but, explants source, carbon source in the medium and the light and temperature conditions are also critical. Embryogenic rice callus has been used as a suitable material for protoplast isolation and plant regeneration (Tsugawa and Suzuki, 2000).

2.5.2 Establishment of organogenesis and embryogenesis

Rice plants can be regenerated through somatic embryogenesis and/or organogenesis. Somatic embryogenesis is the differentiation of plants from haploid and diploid somatic cells through development of characteristic embryological stages without fusion of the gametes.

Plant regeneration from somatic embryos / embryogenic callus has been reported in many variety of rice (cv. Rasi- Ramesh et al., 2009; cv. Basmati-370, JP-5, GNY-53 – Hussain et al., 2010). However, it is necessary to investigate the mechanisms that permit the optimization of the somatic embryos production. Successful plant regeneration via somatic embryogenesis provides a valuable tool for manipulation by utilization of various techniques such as genetic transformation when integrated with conventional breeding programs, which may lead to crop improvement (Stasolla and Yeung, 2003). However, many factors including choice of plant growth regulators and explants are responsible for successful plant regeneration (Khanna and Raina, 1998).