A Seminar Paper on

Breeding Perspective of Anther Culture in Rice

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ABSTRACT

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Anther culture is a technique in which plants are regenerated from microspores via callus formation in *in vitro* culture. It has become a powerful tool for the rapid production of haploid and inbred lines used for obtaining hybrid cultivars. This paper reviewed various aspects of androgenesis and doubled haploid production in rice and its recent applications in plant breeding, genetics and genomics. After reviewing published research reports, it was found that numerous endogenous and exogenous factors affect the success of anther culture in rice. Among the endogenous factors, rice genotype, physiological stage of the rice plant and development state of the pollen is important. Japonica varieties have shown higher androgensis capability than that of indica ones. Indica rice varieties were reported to have anther necrosis, poor callus proliferation and regeneration of albino plantlets. Among the exogenous factors that affect the success of rice androgenesis, temperature/radiation pretreatment of anther, types of culture media and culture conditions are important. On an average, 25% of indica rice are capable of callus formation from anther and up to 80% of the produced calli develop into plants. Larger pollen grains (50-58 µm in diameter) results in better callus formation. For androgenesis, anther pretreatment with temperatures from 8 to 10°C for 8 days have been recommended to be optimal for many varieties of rice. Enhancement of the green plant regeneration from two- to threefold could be possible by the use of irradiation of the ^{137}Cs gamma rays, and the maximum response was elicited with the dose of 15 Gy. Maltose has been identified as a superior source of carbohydrate compared to sucrose for androgenesis in rice. Tryptophan (25 mg/L) and cysteine (40 mg/L) amino acid supplements improve callus induction. Addition of 2.5 mg/L of 2,4-D in the medium increase the callus indication percentage. A mixture of IAA (2 mg/L) and Kin (2.5 mg/L) was reported to induce 87.5% brown calli and 12.5% plantlet in balck rice. Alternating periods of light with different temperatures (12–18 h; 5000–10,000 lux/m^2 at 28°C and 12–6 h; in darkness at 22 °C) rather than continuous light condition are effective for callus formation. Despite the potential of anther culture in rice breeding programs, its actual application is limited due to some factors like high genotypic dependency, low frequencies of callus induction and plant regeneration, and high frequency of haploid plants.

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CHAPTER 1. INTRODUCTION

Rice (*Oryza sativa* L.) is the carbohydrate source for more than half of the world's population. In the last few decades, there has been tremendous increase in rice productivity with the development of high yielding varieties (Mohiuddin, Karim, & Sultana, 2014). Hybrid rice technology came a viable option to manifold the rice yield lately. Though hybrids have yield advantage over inbreds, the farmers of the developing nations don't favor it due to high cost of seed and poor grain quality.

In vitro androgenesis or anther culture is a where the pollen grains are made to switch from their normal pollen developmental pathway towards an embryogenic route (Rukmini Mishra, Gjn, & Rao, 2013; Rukmini Mishra & Rao, 2016). It is also a tool for the rapid recovery of fixed breeding lines in rice (S. K. Tripathy, Lenka, D., Prusti, A. M., Mishra, D., Swain, D., & Behera, S. K., 2019). Haploid and doubled haploid produced through androgenesis have been used in plant breeding for quite a long time as it can shorten the breeding cycle, fix agronomic characters in homozygous state and increase the selection efficiency of useful recessive agronomic traits. *In vitro* culture of doubled haploid indica hybrid lines through androgenesis with higher yield potential and superior grain quality has also been successful to some extent (Rukmini Mishra, Gjn, & Rao, 2013). Recently, doubled haploids production through genome mapping, quantitative trait locus analysis, and genetic mutation have gave the rice breeding program a new momentum (Ambarwati, 2009; Naik et al., 2016; Rout et al., 2016). The advantages of haploid culture, technique especially the anther culture, in the breeding program have been known (Khush & Virmani, 1985; S.K. Raina, 1989; Zapata, 1985). Anther culture shortens the breeding cycle of new varieties and allows early expression of recessive genes. The selection efficiency is considerably increased as the population contains only fixed homozygous lines. Beside the advantages of using the techniques, anther culture has a disadvantages and constraints, i.e., low efficiencies of callus production, low plant regeneration, and high proportion of albino plants produced. Thus, this review focused mainly on various facets of androgenesis and doubled haploid production in rice and its recent applications in plant breeding, genetics and genomics.

Objectives:

(i) To review the factors affecting the success of androgenesis in rice

(ii) To explore the limitations of androgenesis in rice

CHAPTER 2. MATERIALS AND METHODS

This seminar paper is exclusively a review paper. So, no specific methods of studies are followed to prepare this paper. All facts and data were composed and recycled from secondary sources. This seminar paper has been prepared by reciting different books, journals, booklets, proceeding, newsletters, consultancy report which are available in the online libraries of BSMRAU & internet. Some information is collected from BRRI and some private and international agricultural organizations. Maximum necessary supports were taken from internet searching. Finally, this seminar paper was prepared with the consultation of my respective major professor and honorable seminar course instructors.

CHAPTER 3. REVIEW OF FINDINGS

3.1. History of anther culture in rice

The possibility of changing the normal gametophytic pathway of microspores to sporophytic pathway facilitating the haploid plant development through *in vitro* culture was first reported by Guha (1964) on culturing immature anthers of the Solanaceous species *Datura innoxia* (Ruwani, Mayakaduwa, & Siillva, 2018). Haploid plants were successfully obtained from culturing isolated anthers of *Nicotiana* (Bourgin, 1967) . Anther and pollen isolation followed by haploid production in *in vitro* culture has been successful in many other crop species such as rice, wheat, maize, *Brassica*, and pepper (Javed, Ishii, Kamijima, & Misoo, 2007; Razdan, 2003). However, despite the success of embryogenesis in model species such as barley, rapeseed, tobacco, and wheat, etc., the same in economically important crops such as *Arabidopsis*, woody plants, and legume crops, continue to be less successful. Numerous research have been conducted to establish this technique of developing haploids and dihaploids. For the technique to be practically applied in breeding programs, anther or microspore culture should be able to permit production of haploids in very large quantities from almost any species or genotype (Shahnewaz & Miah, 2004). Haploid plant production in rice through anther culture was first reported by Niizeki and Oono (1968). Since then, many studies have been conducted improving various aspects of rice anther culture. In addition to dihaploid development through anther culture, gene transformation has been also tried (Silva, 2009). In Japan and China, where the Japonica rice varieties are mainly in use, anther culture technique has been extensively applied for improving the rice crop due to the amenability of Japonica rice varieties to *in vitro* anther culture (Javed, Ishii, Kamijima, & Misoo, 2007). However, since the anthers of Indiac rice varieties are recalcitrant in nature, their use in androgenesis has been proven difficult and limited. Therefore, the potential of the technique for Indica rice breeding is yet to be fully unraveled (Razdan, 2003).

3.2. Techniques of rice anther culture

Rice anther culture is carried out in two phases in which the initial step is to induce embryo- genic calluses from microspores followed by green plant regeneration from the induced calluses (Javed, Ishii, Kamijima, & Misoo, 2007; Rukmini Mishra & Rao, 2016). For androgenesis to be successful, normal gametophyte formation from microspores should be halted, and microspores are directed toward sporophyte development. Usually, pre-treatments are required to alter the normal pollen development pathway and to trigger the androgenic response. The pretreatments of anthers during the androgenesis process vary among genotypes and varieties. Therefore, there is no single standard

method that can be applied for androgenesis of all varieties of a species. However, some common protocols to be followed during anther culture are well known and documented (Germanà, 2010). Protocol for rice anther culture includes pretreatment given to panicles, surface sterilization and excision of anthers from panicles, and *in vitro* culture of anthers on a specific culture medium under aseptic conditions (Sah, 2008). Response of anthers in culture is usually indicated by the gradual browning of the anther wall tissues and bursting or splitting of the anther to expose the pollen callus. Pollen callus can be expected to be formed in anthers after 3–8 weeks of culture (Razdan, 2003). The second phase is to regenerate green plants from the calluses using appropriate regeneration media (Sah, 2008). The regenerated plants are then transplanted and acclimatized under controlled environmental conditions, and they can be subjected to chromosome doubling using antimitotic agents in order to obtain doubled haploids which can serve as homozygous lines.

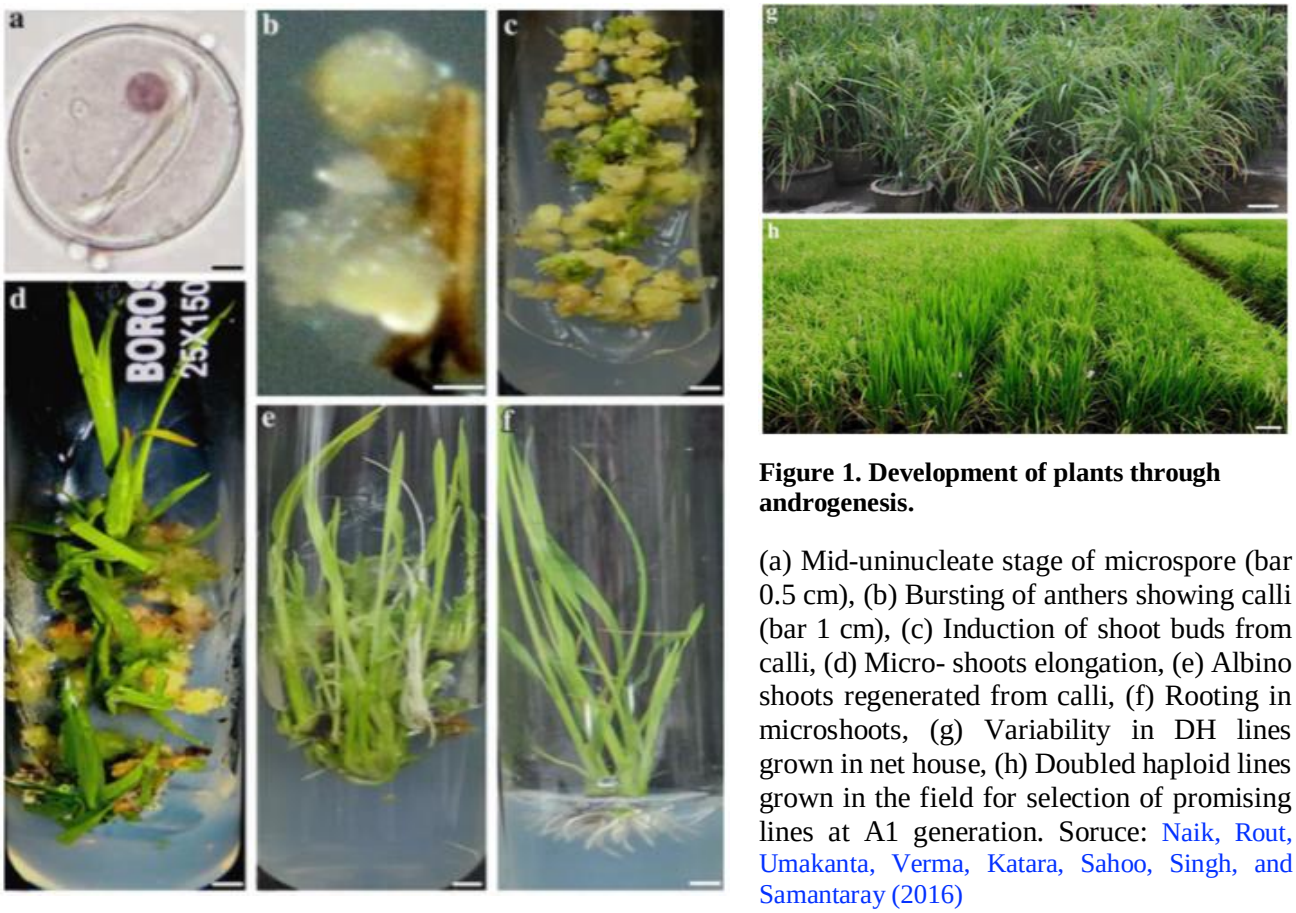

Figure 1. Development of plants through androgenesis.

(a) Mid-uninucleate stage of microspore (bar 0.5 cm), (b) Bursting of anthers showing calli (bar 1 cm), (c) Induction of shoot buds from calli, (d) Micro- shoots elongation, (e) Albino shoots regenerated from calli, (f) Rooting in microshoots, (g) Variability in DH lines grown in net house, (h) Doubled haploid lines grown in the field for selection of promising lines at A1 generation. Soruce: Naik, Rout, Umakanta, Verma, Katara, Sahoo, Singh, and Samantaray (2016)

Rice panicles become are suitable for collection during the booting period, when the microspores are at mid- to late-uninucleate stages. Previous studies have shown that florets having anther length of less than half of the size contain anthers having microspores of mid- to late-uninucleate stage (Niroula & Bimb, 2009). The boots (young panicles still enclosed within the flag leaf sheath) are wiped with a clean muslin cloth moistened with 70% alcohol. The wrapped boots are then cold pre-treated at 10 °C for 8–10 d. The spikelets are then subjected to surface sterilization using 20% commercial bleach (containing 4% sodium hypochlorite) for 5 min followed by thoroughly rinsing three times by sterile de-ionized water. After cytological examination of the microspore stage, 20–25 anthers with microspores at mid-uninucleate to early bi-nucleate stages are dusted over the surface of the media. The inoculated anthers are incubated in dark at (25 ± 1) °C and observations on the anther response to callus induction were recorded starting from 3–4 weeks after inoculation. Then, the calli are transferred onto the regeneration medium and incubated under artificial light (about 2 000 lux) at (25 ± 1) °C for callus regeneration. The green plantlets are then transferred to rooting medium for root formation. The plants with well-formed roots are transferred to pots in a greenhouse (Rukmini Mishra, Gjn, & Rao, 2013).

3.3. Factors affecting the success of rice anther culture

Despite the importance and potentiality of anther culture in plant breeding programs, various factors limit its actual application, including high genotypic dependency, low frequencies of callus induction and plant regeneration, and high frequency of haploid plants (Medhabati, Das, Henary, Singh, & Sunitibala, 2014; A. Sharma, Lal, & Sutradhar, 2017). Indica rice varieties have been reported to show limited response to anther culture since their anthers dries up quickly, and their callus proliferation and albino plantlets regeneration capabilities are poor (Chen et al., 2001), however, japonica varieties showed better performance in androgenesis that the Indica ones (He, Yang, Tu, Yu, & Li, 2006). An array of endogenous and exogenous factors can affect the success of anther culture. The factors are describe in brief in the following sections.

3.3.1. Genotype

Response to anther culture varies differently within species, subspecies, or varieties. *Oryza glaberrima* shows more potential for callusing and regeneration than that of *O. sativa* (Gueye & Ndir, 2010). However, in case of microspore embryogenesis, Japonica types are *in vogue* more responsive to Indica types. Many researchers have reported genotypic specificity within indica subspecies using improved media (Talebi, Rahemi, Arefi, Nourozi, & Bagheri, 2007). Indica cultivars show poor callus growth, poor regeneration ability, and low percentage of albino plants

(Satish K. Raina, 2010). Only 5 of 18 indica cultivars showed pollen callusing and 4 calli differentiate into plants (Guha-Mukherjee, 1973), whereas only 1 of 35 indica cultivars exhibited pollen callusing (Lentini, Reyes, Martínez, & Roca, 1995). Tran and Vuong (2002) also recorded low response for callus induction (3.53%) and plantlet regeneration (1.12%) frequency in indica rice. However, combining high yielding indica rice with high anther culture responding japonica genotype may improve anther culture response (Herath and Bandara 2011). HIgh callus induction frequency from anthers of F1 plants was derived from four crosses of aromatic and improved rice cultivars (Thuan, Tuan, & Ba Bong, 2001). Calli generated from from a F1 hybrid (Bg 90-2: Indica rice/Hu Lo Tao: Japonica rice) showed a higher percentage of green plantlet regeneration than their parents. High callus induction frequencies (30–34%) and low regeneration response in F1s were obtained by R Mishra, Rao, and Rao (2011), while positive relationship for both was noticed by Javed, Ishii, Kamijima, and Misoo (2007) and Shahnewaz, Bari, Siddique, and Rahman (2004). A callus induction frequency of as high as 37.83 % from anther culture of a cross CRMS31B/CRMS24B was reported (Dash, Rao, & Rao, 2014) .

Table 1. Callus induction frequency of anthers of different rice genotypes. Source: Shahnewaz, Bari, Siddique, and Rahman (2004)

Genotypes	Number of anthers inoculated	Number of anthers formed calli	% callus induction	Texture of callus	Color
BR-3	320	11	3.43	Compact	Creamy
BR-7	260	12	4.6	Compact	White
BR-10	350	5	1.42	Compact	White
BR-14	270	7	2.6	Compact	White
BRRI dhan29	310	25	8.06	Compact	White
BR-802-78-2-1-1	280	9	3.2	Compact	White

3.3.2. Physiology of the donor plants

Anthers collected at the beginning of the flowering period respond better, and it declines with the age of plants (Szarejko, 2003). Field-grown plants show superiority over to those grown in the glasshouse or pots (Prem, Gupta, & Agnihotri, 2004; Veeraraghavan, 2007). Since plants with vigorous vegetative growth are not ideal for anther collection, second application and/or foliar spray of nitrogen is avoided. Besides, a favorable day (34°C) and night (25°C) temperatures at booting stage seem to be a determining factor for androgenic embryogenesis (Prem, Gupta, & Agnihotri, 2004).

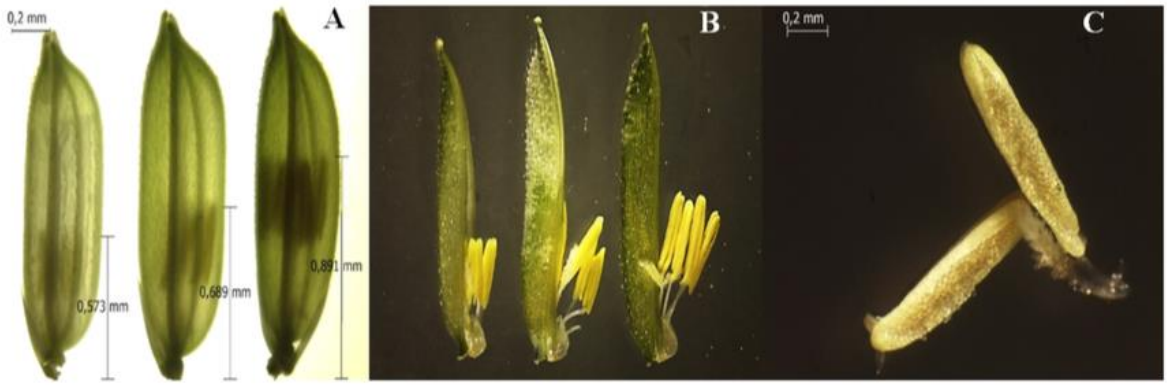

Figure 2. A) Anther position in spikelet; B) The optimal maturity of anther (right position); C) The best anther head of rice for culture. Source: Maharani, Fanata, Laeli, Kim, and Handoyo (2019)

Anthers from the primary tillers are *in vogue* more responsive than secondary tillers (Asif, 2013). Besides, physiological status of anthers from the middle portion of the panicles in boot stage seems to be favorable for callusing and regeneration (Jacquard et al., 2006).

3.3.3. Development stage of pollen

The developmental stage of pollen can enhance anther culture efficiency. In rice, the most suitable stage is the early to mid-uninucleate pollen stage (Datta & Wenzel, 1998), while the older pollens at tetrad stage and after the first pollen mitosis do not respond to culture due to starch deposition leading to differentiation into male gametophyte (Silva, 2009). Such above appropriate condition corresponds to the 3–4 cm distance between the collar of the flag leaf and ligule of the penultimate leaf (Bishnoi et al., 2000). Therefore, the anther culture response in rice should be optimized by assessing the optimum developmental stage of pollen grain by acetocarmine staining. It was observed that only large pollen grains, 50-58 µm in diameter with thin, fight pink colored cell walls seemed to develop into pollen embryos in case of *Oryza sativa* cv. Taipei 309 (Cho & Zapata, 1988).

3.3.3.1. Osmotic stress

Osmotic shock can substitute or be used together with cold treatment for the induction of androgenesis. The treatment of anthers in 0.4 M mannitol solution was found to be effective for inducing androgenesis in microspore cultures of Indica and Japonica varieties (S. K. Raina & Irfan, 1998). Mannitol treatment only promoted androgenesis in anther cultures of variety IR43 from 3 to 33.4%. It is described that when the anthers or isolated microspores are subjected to high osmolarity by incubating in metabolizable carbohydrates for short time, they start divisions during stress treatment and tolerate the following stress conditions (Shariatpanahi, Bal, Heberle-Bors, & Touraev,

2006). Further, regenerability of callus could also be improved markedly by osmotic treatment. It is supposed to regulate the endogenous levels of auxin interacting with abscisic acid affecting the carbohydrate metabolism and thereby trigger both callus initiation and shoot regeneration responses in rice (S.-T. Lee & Huang, 2013).

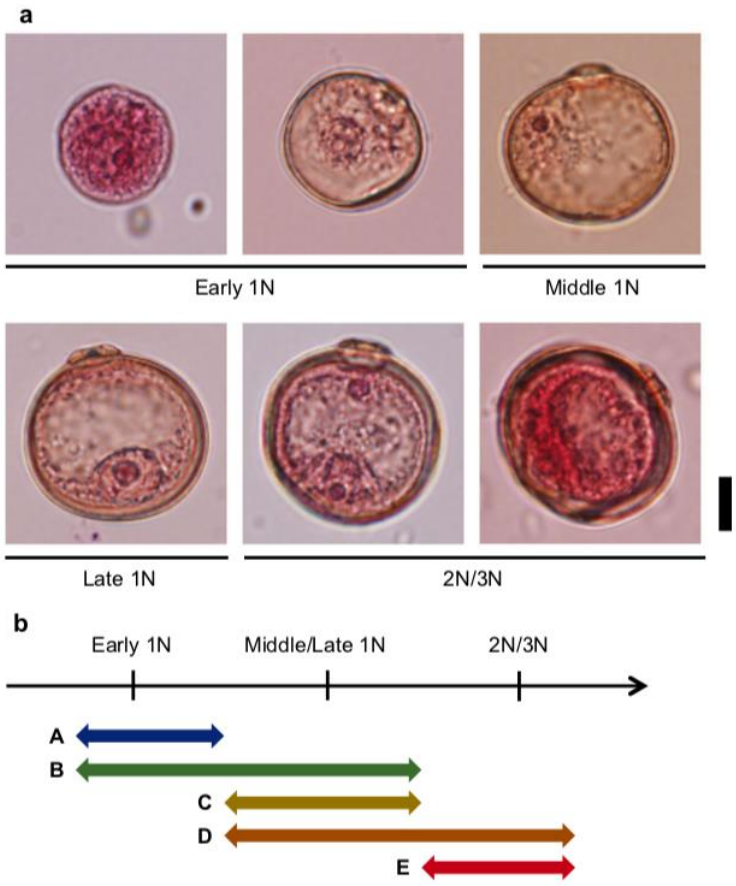

Figure 3. Classification of microspores based on developmental stages.

[a Microspores stained with acetocarmine at different developmental stages. Bar, 10 μm. b Categorization of microspore developmental stages into classes A to E. 1N, 2N, and 3N indicate uninucleate, binucleate, and trinucleate stages, respectively. Source: Kanaoka et al. (2018)]

3.3.4. Pre-treatment

3.3.4.1. Temperature pretreatment

Most frequently used effective method of pretreatment for rice anther culture is the low-temperature application. Harvested rice panicles are given a cold shock treatment prior to the culture. However, the temperature and duration vary with the variety. Cold pretreatment given to rice anthers is known to enhance the androgenesis potential by delaying the degeneration of microspores and anther wall tissue in rice (Bhojwani, Pande, & Raina, 2001; Silva, 2009). Matsushima, Kikuchi, Takaiwa, and Oono (1988) reported that a pretreatment at 10 °C for 10–30 days was necessary to induce

sporophytic divisions in microspores of the Japonicas. Generally, temperatures from 8 to 10°C for 8 days have been recommended to be optimal for many varieties of rice (Zapata-Arias, 2003). Panicle pretreatments longer than 11 days tend to increase albino production (Gupta & Borthakur, 1987). A brief exposure to high temperature (35°C for 10 min) before the cold treatment can enhance callus induction although it can affect green plant production (Reddy, Leelavathi, & Sen, 1985) .

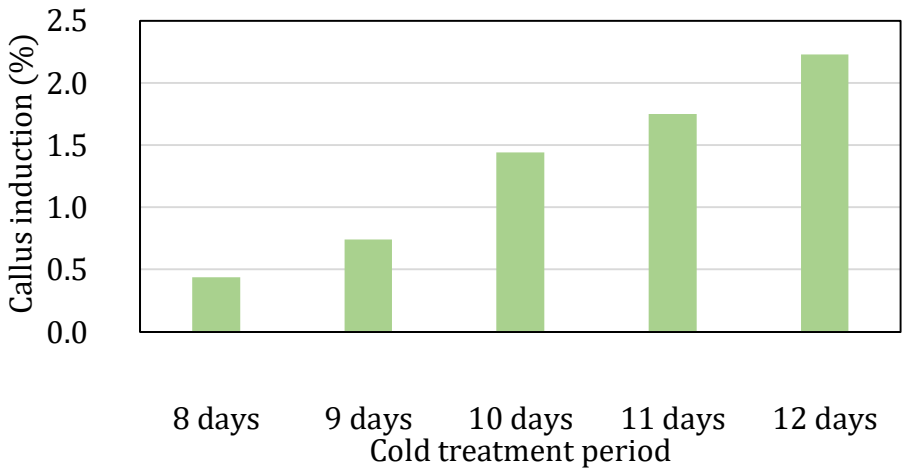

Figure 4. Effect of cold treatment (4 °C) on callus induction of rice. Source: V. Sharma et al. (2020).

3.3.4.2. Sugar starvation

Not only in rice but also in many other crop species such as tobacco, wheat, and barley, sugar starvation has been found effective in induction of embryogenesis (Shariatpanahi, Bal, Heberle-Bors, & Touraev, 2006). However, cold pretreatment could be partially substituted by subjecting microspores for sugar starvation for 3 days during androgenesis of Indica rice (Ogawa, Fukuoka, & Ohkawa, 1995) . S. K. Raina and Irfan (1998) also confirmed that sugar starvation could be applied for Indica and Japonica rice in obtaining high-frequency embryogenesis and plantlet regeneration. Many changes induced in starved microspores at cytoplasmic and nuclear levels have been described in detail by other researches (Shariatpanahi, Bal, Heberle-Bors, & Touraev, 2006).

3.3.4.3. Irradiation

Penetration of irradiation varies with the species and dependent pollen morphology and the thickness of the pollen wall (Savaskan, 2002). The stimulation of green plant regeneration from rice anther culture with the application of gamma rays at the dose of 20 Gy was effective (Chen, Wang, Lu, Shen, Afza, Duren, & Brunner, 2001) . Enhancement of the green plant regeneration from two-

to threefold could be possible by the use of irradiation of the ^{137}Cs gamma rays, and the maximum response was elicited with the dose of 15 Gy (Mkuya, Si, Liu, & Sun, 2005).

3.3.5. Culture media

The two main phases of anther culture in rice, callus induction and shoot regeneration, require different nutrient regimes and growth regulators. The culture medium that best supports callus induction is often not suitable for regeneration. Therefore, the transfer of callus onto a suitable regeneration medium must be done at an appropriate time. Since the callus induction potential of a given rice variety is largely determined by the genetic makeup, significant levels of improvement in anther response cannot be expected by manipulation of nongenetic factors such as the culture medium. Nevertheless, the best responsive nutrient requirements must be chosen as an initial step in order to optimize anther culture, particularly if they are low responding Indica varieties (Silva, 2009). The most commonly used basal media for anther culture are N6 medium (Chu, 1978), MS medium (Murashige & Skoog, 1962), and B5 medium (Gamborg, Miller, & Ojima, 1968). Generally, basal N6 medium supplemented with plant growth regulators has been used extensively in cereal anther culture to initiate callus. Macronutrients of culture media comprises mainly of carbon and nitrogen sources. Embryogenic and morpho- genic responses are elicited by supplementing the basal media with appropriate plant growth regulators at effective concentrations. Physical state of the culture medium and also culture maintenance conditions are equally important for the success of rice anther culture.

3.3.5.1. Carbohydrate source

A carbohydrate source is essential in tissue culture media because it serves as the main source of energy to the cultured explant tissue. Carbohydrates are also important as osmotic agents. In rice anther culture, osmotic pressure in the medium is generally regulated by applying the carbohydrate source to the medium at a particular concentration. Very high concentrations when used during the later stages of culture seem to be deleterious for cereals (Germanà, 2010). The type of carbon source directly influences the anther response. Although many early studies have used sucrose as the standard carbon source, different sources have also been tested and proven effective for cereals. In cereals, it has been shown that maltose is a better carbohydrate source compared to sucrose for androgenesis.

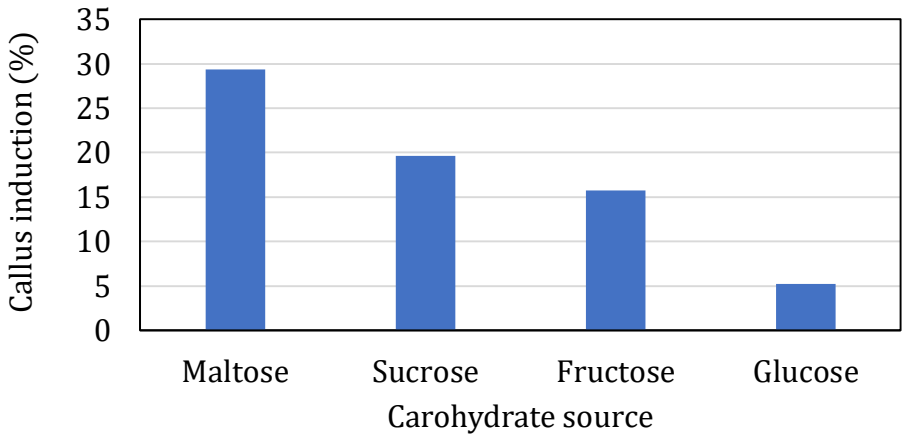

Figure 5. Effect of sources of carbohydrate on the callus induction of rice androgenesis. Source: Naik, Rout, Umakanta, Verma, Katara, Sahoo, Singh, and Samantaray (2016)

Anther culture efficiency and green plant formation of highly recalcitrant Indica rice varieties could be improved significantly when sucrose was replaced by maltose (Bhojwani, Pande, & Raina, 2001), sucrose produced only 1 out of 23 Indica rice varieties responded with pollen callusing and green plant production on N6 medium provided with 146 mM sucrose (Lentini, Reyes, Martínez, & Roca, 1995) . When sucrose was replaced by equimolar amount of maltose, callus induction response improved from 6.3% to 10.1% and green plant regeneration from 0.6–1%. In another report it was observed that 20% maltose used for microspore isolation and 9% maltose used for culturing produced a genotype-independent plant regeneration response (Mejza, Morgant, DiBona, & Wong, 1993). In other cereals such as wheat, maize, and barley, maltose promoted direct embryogenesis from cultured pollen. Sucrose is rapidly broken down into glucose and fructose. Since the microspores are sensitive to fructose, sucrose is shown to be toxic during androgenesis. This also causes depletion of sucrose in the medium with time (Bhojwani, Pande, & Raina, 2001). Comparatively, long-term availability of maltose in the culture medium has been detected due to the slow rate of hydrolysis.

3.3.5.2. Nitrogen source

In culture media, inorganic nitrogen is usually supplied in the form of nitrate and/or ammonium ions. The ratio of the two nitrogen sources NO_3^-:NH_4^+ has been found to be critical for the success of anther culture in rice (Silva, 2009). The N6 basal medium which is most widely used for rice anther culture has been formulated with both these sources of nitrogen at specific concentrations. However, Indica rice varieties perform much better when lower concentration of NH_4^+ ions than normal is used in the medium (Bhojwani, Pande, & Raina, 2001). He_2 medium were found be more effective than N6 medium in a culture of eight Indica rice varieties (Reddy, Leelavathi, & Sen,

1985). He_2 medium is derived from the N medium by reducing NH_4^+ concentration to half strength. In Korea, N -Y 6461 medium which is similar to N6 except that the $(NH_4)_2SO_4$ concentration is reduced from 3.5 to 1.5 mM has been recommended for Indica-Japonica hybrids (Javed, Ishii, Kamijima, & Misoo, 2007). A significant improvement in anther culture could be made in Indica x Indica F1 hybrids using a medium with high KNO_3 and NH_4^+ ions completely replaced by an organic source of nitrogen, casein hydrolysate, at 50 mgL^{-1} (S.K. Raina, 1989). Ogawa, Fukuoka, and Ohkawa (1995) studied the effect of nitrogen source on androgenesis in another Indica variety IR24 using R-2 medium as the control. R-2 has been formulated with 40 mM KNO_3 and 2.5 mM $(NH_4)_2SO_4$. When 20 mM KNO_3 was combined with the amino acid 5 mM alanine, superior green plant regeneration could be achieved. In rice anther culture, amino acids such as proline and glutamine added to the culture media have been able to increase the rate of callus induction from cultured anthers while avoiding the degeneration of anther wall tissue (Germanà, 2010).

Table 2. Effect of amino acid supplemented media on callus induction frequency

Amino acids	Anther inoculated	Callus induced	Callus induction (%)
Proline (560 mg/L)	195	14	7.13
Glutamine (500 mg/L)	206	20	9.7
Tryptophan (25 mg/L)	232	19	8.19
Cysteine (40 mg/L)	240	21	8.74
Tryptophan (25 mg/L) + Cysteine (40 mg/L)	239	30	12.55
Total	1112	104	9.28±0.020

Source: V. Sharma, K. Gupta, Ku. Sharma, Sharma, K. Salgotra, K. Singh, and Sharma (2020)

3.3.5.3. Plant growth regulators

Plant growth regulators have been widely investigated in anther culture. Supplementing *in vitro* culture media with effective growth regulators (auxins, cytokinins, or a combination of these) as appropriate is crucial for the success of androgenic response particularly from recalcitrant genotypes (Germanà, 2010). The growth regulator, 2,4-dichloro-phenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) alone or with kinetin in the culture medium seem to be the major determinants for embryogenic callusing from rice anthers (Xa & Lang, 2011). 2,4-dichlorophenoxy acetic acid (2,4-D) is commonly used in the first phase of rice anther culture, and 2,4-D provided at fairly high concentrations (2 mgL^{-1}) has produced improved rates of callus induction of up to 15% in some genotypes (S. K. Raina & Zapata, 1997). Also, applicability of some other auxins

such as naphthalene acetic acid (NAA), phenyl acetic acid, picloram, and dicamba alone or in combination with 2,4-D has been tested for improving androgenic response. Neither 2,4-D nor NAA alone in the media can support regeneration, but the use of cytokinins such as kinetin (Kn) and 6 benzyl aminopurine (BAP) with NAA is required for regeneration. A combination of 0.5 ppm indole acetic acid (IAA) and 2.0 ppm facilitates the germination of androgenic embryos (S. K. Tripathy et al., 2019). In addition to the growth regulator combination, the auxin/cytokinin balance plays important role in effective androgenesis. 2,4-D (2 mgL^{-1}), picloram (0.07 mgL^{-1}), and kinetin (0.5 mgL^{-1}) was favorable for enhancing the anther response in a large number of genotypes (Lentini, Reyes, Martínez, & Roca, 1995). Further, the type of auxin and its concentration determine the microspore development pathway. For example, the use of 2,4-D favored callus formation, whereas indole-3-acetic acid and NAA promoted direct embryogenesis from cultured anthers without an intervening callus phase (Germanà, 2010). Although high levels of 2,4-D were useful for increasing callus production, it has proven to have a negative effect on the next phase of culture which is regeneration from callus, particularly from recalcitrant rice genotypes (Guha-Mukherjee, 1973). A lower level of 2,4-D (0.5 mgL^{-1}) in combination with the milder auxin NAA (2.5 mgL^{-1}) and kinetin (0.5 mgL^{-1}) has been used effectively during both phases (Shahnewaz & Miah, 2004). This suggests that the use of 2,4-D in the callus induction medium needs to be regulated with a compromise reached between callus induction and regeneration efficiency (S. K. Raina & Zapata, 1997).

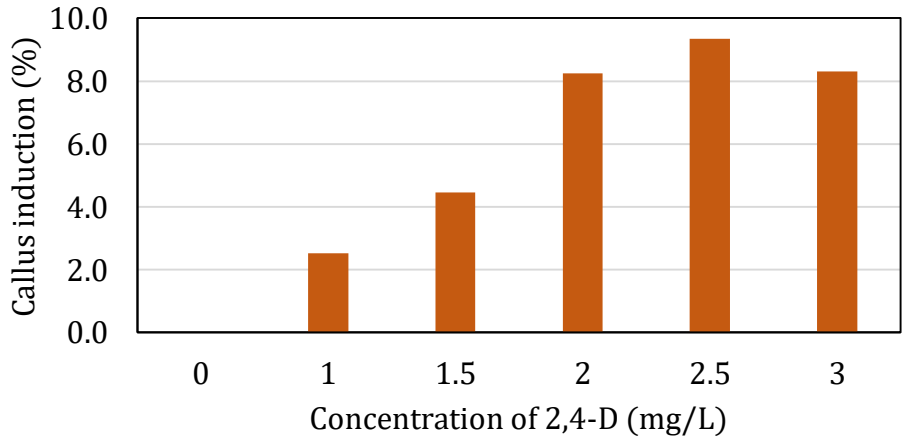

Figure 6. Concentration of 2,4-D on callus induction of rice anther. Source: V. Sharma, K. Gupta, Ku. Sharma, Sharma, K. Salgotra, K. Singh, and Sharma (2020).

Table 3. Optimization of plant regeneration from indica black rice anther calli. Source: Maharani, Fanata, Laeli, Kim, and Handoyo (2019)

PGR's combination	Brown calli	Plantlet	Root
0 mg/L IAA + 1.0 mg/L Kin	75	0	25
0 mg/L IAA + 1.5 mg/L Kin	0	0	0
0 mg/L IAA + 2.0 mg/L Kin	0	0	0
0 mg/L IAA + 2.5 mg/L Kin	100	0	0
1 mg/L IAA + 1.0 mg/L Kin	75	0	25
1 mg/L IAA + 1.5 mg/L Kin	87.5	0	12.5
1 mg/L IAA + 2.0 mg/L Kin	0	0	0
1 mg/L IAA + 2.5 mg/L Kin	100	0	0
2 mg/L IAA + 1.0 mg/L Kin	50	0	50
2 mg/L IAA + 1.5 mg/L Kin	87.5	0	12.5
2 mg/L IAA + 2.0 mg/L Kin	0	0	0
2 mg/L IAA + 2.5 mg/L Kin	87.5	12.5	0

Embryo formation (10.8%) was reported to be markedly increased by abscisic acid in the callus induction media or its treatment during the cold pre-treatment period (Ghaemi, Sarrafi, & Alibert, 1994). Ethylene is also produced in plant cell due to the presence of auxin (Dewi & Purwoko, 2016), sucrose (Lal, Shashidhar, Godwa, & Ashok, 2014), or calcium (S. Y. Lee, 2003) in the callus induction medium. Profused callusing may be due to the inhibitory effect of endogenously produced ethylene from excised anthers (S.-T. Lee & Huang, 2013). AgNO3 (Lentini, Reyes, Martínez, & Roca, 1995) and polyamines (Cha-um, Srianan, Pichakum, & Kirdmanee, 2009) also affect anther culture response in rice through inhibition of ethylene synthesis. Putrescine application to the callus medium also increases the frequency of green/ albino regeneration frequency both in japonica and indica variety.

3.3.5.4. Physical state of the medium

Usually, rice anthers are cultured on solid media. However, higher percentage of necrosis of anther tissue was reported found when cultured on solid media and it was a better in liquid media (Lentini, Reyes, Martínez, & Roca, 1995). Liquid culture media are able to supply the anthers with an improved access to nutrients and plant growth regulators, and also toxic and degenerated material can be readily dispersed. During the culture of anthers from Indica×Basmati rice on liquid media, several fold increments in green plant regeneration comparable with the rates reported for Japonica rice varieties/hybrids could be obtained (Bishnoi, Jain, Rohilla, Chowdhury, Gupta, & Chowdhury, 2000). However, since the rice anthers tend to settle at the bottom of the liquid cultures, this would affect respiration and result in loss of viability of the explants. When the liquid culture media was added with substances such as Ficoll, it was possible to avoid sinking of anthers due to the increased buoyancy, and therefore viability could be maintained (Silva, 2009). In principle, the solidifying

agent should not carry any nutritional effect. Agar is in extensive use as the gelling agent of solid culture media. However, more reproducible results have been obtained with the use of Gelrite. Starch also has been used for solidification despite the nutritional effects and its dissociation into sugar (Foroughi-Wehr & Wenzel, 1993). Some have found improved response by embedding anthers in agarose than culturing on semisolid or liquid media (Gill, Kaur, Sindhu, Bharaj, & Gosal, 2008).

3.3.6. Culture incubation conditions

Culture temperature plays an important role in plant tissue culture. Anther cultures are usually incubated at the temperature range of 24–27 °C. Nona Bokra and Pokkali (Indica varieties), callus induction frequencies and plant regeneration were improved when cultures were incubated at alternating temperature regime of 30/20 °C (14/10 h) instead of constant incubation at 25°C (Javed, Ishii, Kamijima, & Misoo, 2007). Light regulates morphogenesis of cultured pollen and specifically darkness (low intensity of light) or alternating light and dark conditions can be preferable for embryogenic induction. Alternating periods of light with different temperatures (12–18 h; 5000–10,000 lux/m^2 at 28°C and 12–6 h; in darkness at 22 °C) was found to be effective in callus formation (Vasil, 1973). Regeneration phase requires even more specific incubation conditions to achieve success. Shoot regeneration from scutellum-derived callus of Indica rice was stimulated by applying osmotic stress conditions. Osmotic stress was created in tissues by altering the water content of the medium with the use of agarose and mannitol or by partial desiccation of callus (Zapata-Arias, 2003). It is possible to expect similar stimulatory effects in anther-derived callus also. With osmotic stress, water content in the calluses is reduced, thus converting the callus tissues into more compact structures with better embryogenic and regeneration potential (Jain, Jain, & Wu, 1996). The composition of the atmosphere in the culture vessel has not been thoroughly studied despite its importance shown with tobacco (Germanà, 2010). Explant density and explant orientation in the culture medium also have been found to be critical in anther culture (Germanà, 2010; Razdan, 2003).

Table 4. Effects of basal media and culture temperatures on callus induction and plant regeneration in anther culture of salt tolerant indica rice cultivars, Nona Bokra and Pokkali. Source: Javed, Ishii, Kamijima, and Misoo (2007)

Cultivar name	Culture temp.	CIM[1]	N_A	Callus induction		PRM[4]	N_C	Plant regeneration		
				RA[2]	CP[3]			GS[5]	AS[6]	GSP[7]
Nona Bokra	25 °C	SK-I	800	2.5	3	SK-II	24	0	0	0
		N6	600	1.5	2	N6	12	0	0	0
		DKN	600	1	1.5	DKN	9	0	0	0
	32/20 °C	SK-I	1600	3	3.9	SK-II	62	3.2	12.9	0.1
		N6	600	2.3	3	N6	18	0	0	0
		DKN	600	1	2	DKN	12	0	0	0
Pokkali	25 °C	SK-I	800	7.5	9	SK-II	72	5.6	8.3	0.5
		N6	600	4	5.2	N6	31	0	3.2	0
		DKN	600	1	3.5	DKN	21	0	0	0
	32/20 °C	SK-I	1600	12.8	24.3	SK-II	292	17.5	14.4	4.3
		N6	600	4.8	7.2	N6	43	0	0	0
		DKN	600	1.5	6	DKN	36	0	0	0

[Abbreviations: [1]Callus induction media; [2]Rate of responding anther × (No. of anthers yielded calli/No. of anthers cultured) ×100; [3]Callus productivity × (Total no. of calli yielded/No. of anthers cultured) ×100; [4]Plant regeneration media; [5]Regeneration frequency of green shoot × (No. of green shoots/No. of calli cultured) ×100; [6]Regeneration frequency of albino shoot × (No. of albino shoots/No. of calli cultured) ×100. [7]Green shoot productivity × (No. of green shoots regenerated/No. of anthers cultured) × 100; N_A: number of anther; N_C: number of callus]

3.4. Production of double Haploids and their viability

The androgenesis is based on the principle of arresting the development of male gametophytes (pollen grains) and to force them to follow somatic pathway. Under certain culture conditions in anther culture, the pollen grain (haploid) induce, divide and double the chromosome number while reversing the process of gametophyte (early- to mid-uninucleate) to sporophytic stage (Ruwani, Mayakaduwa, & Siillva, 2018). Ideally, the plants developed from anther culture can be considered as haploids as they are arisen from haploid microspores. However, the actual plants resulted during the regeneration could be a mixture of haploid, diploid, or mixoploid (Foroughi-Wehr & Wenzel, 1993). Occurrence of non-haploids can be due to different malformations.

Table 5. Haploid and diploid nature of plantlets derived from anther culture of the Thai rice cultivars Pathumthani, KDML105 and Homjan. Cha-um, Srianan, Pichakum, and Kirdmanee (2009)

Cultivars	Total plantlet	Haploid plantlet		Diploid plantlet	
		Number	Percentage	Number	Percentage
PT1	47	32	68.1	15	31.9
KDML105	41	29	70.7	12	29.3
HJ	79	62	78.5	17	21.5

When the vegetative and generative nuclei are not separated by cell wall formation, non-haploids could be originated (Germanà, 2010). Colchicine treatment of apical meristem of anther-derived haploid plants produce DH cells in small sectors which normally produce DH seeds. Microspore derived calli often show aneuploids, dihaploids and polyploids (Debata & Patnaik, 1989). Since colchicine treatment produce mixoploids and polyploids resulting in chimeric plants and low seed set, it is usually avoided at whole plant level in rice. However, by applying colchicine during early stages of androgenesis, these problems can be alleviated (Castillo, Cistué, Vallés, & Soriano, 2009) and increased the frequency of DH development in the shortest time.

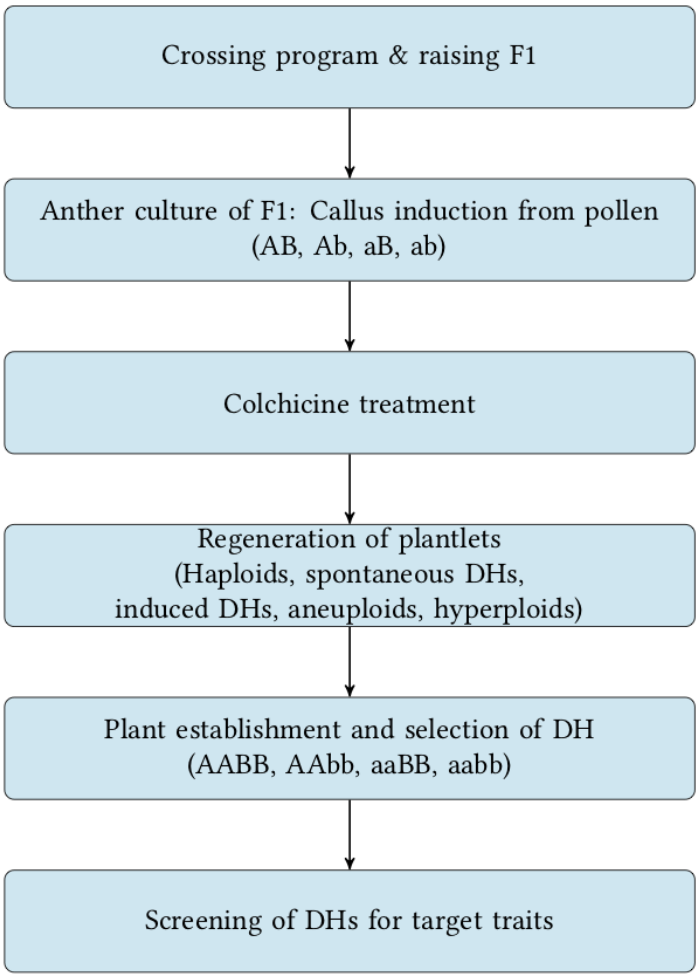

Figure 7. Pathway of producing double haploids in rice. Redrawn from S. K. Tripathy, Lenka, D., Prusti, A. M., Mishra, D., Swain, D., & Behera, S. K. (2019).

Supplementation of 0.2-0.5 g colchicine L^{-1} for 24-48 h incubation results in viable double haploid embryo-like structure formation which on transfer to colchicine-free regeneration medium induces as high as 65-70% viable DH plantlets and it was twice in comparison to spontaneous doubling (Premvaranon, Vearasilp, Thanapornpoonpong, Karladee, & Gorinstein, 2011). Alternatively, the

anther culture derived haploid plants may be immersed in 0.1% colchicine and 2% dimethyl suphoxide (DMSO) solution for 4-5 h (Zapata-Arias, 2003) or colchicine (1.25 mM) and oryzalin (25 μM) solution for 12 h (Chen, Wang, Lu, Shen, Afza, Duren, & Brunner, 2001) to obtain mirror image of haploid set of chromosomes in DH lines. In rice, haploid set of chromosomes in microspores also become spontaneously doubled under suitable culture conditions. Higher (50-60%) spontaneous chromosome doubling (50-60%) (endoreduplication) in culture (Germanà, 2010). A maximum of 60% spontaneous DH green plantlets could be recovered through mutagenesis in Japonica rice (S. Y. Lee, Kim, & Kwon, 2004).

Colchicine treatment (30 mg L^{-1}) has significant stimulating effect on regeneration of higher frequency of green DH mutant plantlets from anther culture of rice varieties (Chen, Wang, Lu, Shen, Afza, Duren, & Brunner, 2001) . Thus, colchicine treatment may be omitted for DH production in rice. It successful to recover DH lines from hybrids *O. sativa* x *O. glaberrima* (Jones, Dingkuhn, Aluko, & Semon, 1996). Higher number of DH lines were recovered in Indica rice (var. 'Gobind', 'HKR120', 'Basmati 370' and 'Taraori Basmati') and an Indica-Basmati hybrid via colchicine treatment (0.1%, w/v). Several researchers reported that they could recover a few promising uniform and stable DH lines from Indica rice hybrid using morphological and molecular markers (Naik, Rout, Umakanta, Verma, Katara, Sahoo, Singh, & Samantaray, 2016; Rout, Naik, Ngangkham, Verma, Katara, Singh, & Samantaray, 2016). Chromosome counting combined with segregation analysis to distinguish diploids, haploids and double haploids plants by suggested some researchers (Bhattacharya et al., 2014).

Table 6. List of rice varieties released using double haploid breeding

Country	Rice variety	Traits	Reference
India	IR64	Blast resistance	Ambarwati (2009)
India	CRHR32 (hybrid)	Long duration, higher yield	Rout, Naik, Ngangkham, Verma, Katara, Singh, and Samantaray (2016)
India	BS6444G (hybrid)	Higher yield	Naik, Rout, Umakanta, Verma, Katara, Sahoo, Singh, and Samantaray (2016)
China	Guan 18, Gan Xhao Xian No. 11	Early maturity; good quality and disease resistance	Zhu and Pan (1990)
South Korea	Hwacheongbyeo, Joryeongbyeo, Hwajinbyeo	Brown plant hopper, stripe virus, blast and bacterial blight resistance	Y. T. Lee et al. (1989)
India	CR Dhan 801, Phalguni	Leaf blast, gall midge resistance	NRRI (2010)
India	CR Dhan 10, Satyakrishna	Resistant to neck blast, sheath rot	NRRI (2008)
Chian	Huayu 15	Lodging and diseases resistance; good grain quality	Shouyi and Shouyin (1991)
South Korea	PSBRc 50 Bycol (IR 51500-AC- 11-1)	Salt tolerance	Senadhira et al. (1996)
Phillippines	PSBRc 50 Bycol (IR51500AC-11- 1)	Salt tolerance	Senadhira et al. (2002)
India	Janka	Drought tolerance; good grain quality	Pauk, Janeso, and Simon-Kiss (2009)
India	Abel	Cold tolerance at early stage	Pauk, Janeso, and Simon-Kiss (2009)
Japan	Joiku N 394, Hirohikari, Hirohonami, AC. No.1, Kibinohana	Cold tolerance and good taste	Singh (1998)
China	Huayu I, Huayu II, Xin Xiu, Late Keng 959, Tunghua 1, Tunghua 2, Tunghua 3, Zhonghua 8, Zhonghua 9, Hua 03, Huahanzao, Huajian 7902, Tanghuo 2, Shanhua 7706, Huahanzao 77001, Noll	High yield, superior grain quality; blast and bacterial blight resistance	Yang and Fu (1989)
Hungary	Dama	High yield, resistant to pyricularia, good cooking quality	Heszky and Simon-Kiss (1992)
China	Milyang 90	Good grain quality; brown plant hopper and stripe virus resistance	Chung (1987))
India	Parag 401	Superior grain quality and resistant to iron chorosis	Patil, Nerkar, Misal, and Harkal (1997)
China	Hua 03	High protein content (13.7%)	Yang and Fu (1989)
India	Risabell	High milling, good cooking quality and resistant to blast	Pauk, Janeso, and Simon-Kiss (2009)

3.5. Limitations of rice anther culture

Induction of haploids in rice is associated with a number of constraints. Fine tuning of anther culture process addressing the constraints is required in order to use this technique equally well for breeding of Japonica and Indica rice (S. Tripathy, 2018; S. K. Tripathy, Lenka, D., Prusti, A. M., Mishra, D., Swain, D., & Behera, S. K., 2019). Although this technique has been used to produce haploids from many species, its success cannot be proven in respect of all genotypes of a crop species (Weigt, Kiel, Nawracala, Pluta, & Lacka, 2016). Particularly when it comes to the anther culture of Indica rice, the response remains extremely variety or genotype specific (Bhojwani, Pande, & Raina, 2001). Early anther necrosis, poor callus proliferation and albino-plant regeneration are currently recognized as the major problems in Indica rice varieties (Khatun, Islam, Ara, Tuteja, & Miah, 2012). The problem is further aggravated because anther culture response is affected even by the growing season (Germanà, 2010). At least 15,000 anthers need to be inoculated for *in vitro* culture to obtain regeneration of 150 plantlets (Huan, 1995). A higher percentage of regenerated plants in anther culture are albinos (Talebi, Rahemi, Arefi, Nourozi, & Bagheri, 2007). Gueye and Ndir (2010) could recover 93 regenerants out of which 79 were albinos. The basic cause for albino plant production is breakage of DNA in plastids and nuclei (Kumari, Clarke, Small, & Siddique, 2009). This problem is more serious in Indica rice than Japonica rice. As per Asaduzzaman et al. (2003), albinism can be reduced by shortening the culture period. Frequency of albinism among regenerated is controlled by QTL on chromosome 9 and 10 (Yamagishi et al., 1998). This problem can be minimized by early transfer of anther culture-induced calli into regeneration media, a low temperature incubation (<26 °C) or medium modification for callus induction and plant regeneration Under *in vitro* conditions, many of the anthers fail to grow in culture and thus repress the pollen from forming calluses. Some reasons for failure are the early abortion of pollen and even in situations where pollen starts to divide and produce callus and necrosis or cell death occurs very early during callus proliferation. There is also a degree of uncertainty associated with the ploidy of the resulting callus tissue as it can comprise a chimera of diploid, tetraploid, and haploid cells. Another problem that seriously affects the anther culture of cereals is the formation of albino plants during regeneration, and this can be identified as the most limiting step in the anther culture process (Torp & Andersen, 2009). Detailed investigations of proplastids and the plastid genome of the regenerated albino plantlets revealed that albinism is mainly due to incomplete formation of the membrane structures and different blockages in the plastid development (Foroughi-Wehr & Wenzel, 1993).

CHAPTER 4. CONCLUSION

There are several breeding methods available now-a-days which broaden the genetic variation and recover the desirable gene combinations. A single anther of a intervarietal/interspecific hybrid that carries thousands of pollen grains with different genotypes, can be induced to form stable DH plantlets within a very short time.

Rice breeding through androgenesis has achieved a tremendous advancement. Advanced technologies have been developed for production of double haploid rice plants through anther culture within short time as opposed to conventional breeding. An anther culture program with optimized culture condiiton can accelerate the breeding process for the isolation of plant types with higher yield, disease resistance, and improved quality traits. Besides, an optimized technique can be used to detect and fix desirable recessive traits induced through mutation or spontaneous gametoclonal variation. Many factors, such as, genotype, physiological status of source plant, pollen stage, media recipes, including hormonal supplementation, pre-treatment of anthers, and culture conditions determine the success of anther culture. However, these factors are genotypic and varietal specific for the mother plants and different sets of conditions are required for different rice genotype.

Despite the shortest possible breeding cycle, rice androgenesis some limitations. High genotypic dependency, low frequencies of callus induction and plant regeneration, and high frequency of haploid plants are the major constraints of this method.

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