Chapter 1

Anther Culture as a Supplementary Tool for Rice Breeding

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Abstract

There is a timely need to harness biotechnology and related tools to support conventional breeding strategies, overcoming the limitations in rice production and improving quantity and quality as well as climatic and disease stress tolerance of the crop. Anther culture allows immediate fixation of homozygosity through diploidization of regenerated haploid plants and therefore serves as an efficient path for inbred line development. Anther culture has been successfully used to hasten the breeding programs in several crop species including rice. However, associated constraints still prevent the realization of its full potential. Even though anther culture technique has been effective for Japonica rice breeding, applicability for Indica rice remains limited mainly due to inherent recalcitrant genetic background. Constraints associated with Indica rice can be identified as early anther necrosis, poor callus induction and proliferation, extremely low green plant regeneration and frequent albinism. Success of androgenesis is determined by factors such as genotype, physiological status of donor plant, pollen development stage at culture, composition and physical status of culture media, culture incubation conditions and anther pretreatments. This chapter has detailed out the scope for improving the applicability of anther culture technique on rice in order to develop it as a supplementary breeding tool.

Keywords: callus induction, plant regeneration, microspores, ploidy, homozygous lines

1. Introduction

From ancient times the crop rice has served the human population as a staple food. Due to the steep increase in human population, rice growers need to increase the production as well. This has become more difficult due to the limitations in available resources. Therefore, other

than the conventional strategies, it is a timely need to harness biotechnology and related tools to overcome not only the productivity barrier but also the production efficiency, quality of the product, and abiotic and biotic stress tolerance of the crop. Among a number of modern biotechnological tools to improve rice crop, anther culture plays a useful role.

Anther culture can be considered as a technique for the rapid development of fully homozygous lines. Therefore, anther culture technique provides an efficient alternative to the conventional inbred line development which is usually achieved through several cycles of inbreeding. Even though anther culture has been efficiently used as a supplementary breeding tool with Japonica rice varieties, application of this technique for Indica rice varieties is limited due to their inherent recalcitrant genetic background. Limitations of androgenesis in Indica rice result from early anther necrosis, poor callus induction and proliferation, remarkably low green plant regenerability, and frequent albinism [1, 2]. Success of the technique is determined by numerous factors such as genotype and physiological status of donor plant, pollen development stage at culture, anther wall factors, composition of culture media including nutritional sources and growth regulators, physical status of culture media, and anther pretreatments [1, 3, 4].

This chapter serves as an insight to the practical aspects of anther culture technique for it to be fully exploited for improving rice breeding.

2. Technique of anther culture

For androgenesis to be successful, normal gametophyte formation from microspores should be halted, and microspores are directed toward sporophyte development. Usually, pretreatments are required to alter the normal pollen development pathway and to trigger the androgenic response. The specific pretreatments for androgenesis that are required by different species and also varieties within species are quite variable. Therefore, a single standard method cannot be generalized for androgenesis for a given species or even a variety. However, some common protocols to be followed during anther culture are well known and documented [4].

Rice anther culture is carried out in two phases in which the initial step is to induce embryogenic calluses from microspores followed by green plant regeneration from the induced calluses [5]. 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 [6]. 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 [2]. The second phase is to regenerate green plants from the calluses using appropriate regeneration media [6]. 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.

3. Historical trajectory of anther culture

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 reference [7] on culturing immature anthers of the Solanaceous species *Datura innoxia*. Reference [8] successfully obtained haploid plants from culturing isolated anthers of *Nicotiana*. Since then, haploid development using *in vitro* culture of anthers and isolated pollen has been successful with many other crop species such as rice, wheat, maize, *Brassica*, and pepper [2, 5]. Although microspore embryogenesis has been effective with model species such as barley, rapeseed, tobacco, and wheat, some other species that are scientifically or economically important, such as *Arabidopsis*, woody plants, and legume crops, continue to be less responsive for the technique. Extensive research has been performed in order to make this important technique of developing haploids and dihaploids more robust. 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 [9].

Haploid plant production in rice through anther culture was first reported by reference [10]. Since then, many studies have been conducted improving various aspects of rice anther culture. Other than utilizing anther culture technique directly for dihaploid development, recently applications have been expanded to facilitate other biotechnological approaches such as gene transformation [3]. 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 [5]. However, the use of this technique as a tool for Indica rice breeding has been extremely limited due to the inherent recalcitrance associated with Indica varieties. Therefore, the potential of the technique for Indica rice breeding is yet to be fully unraveled [3].

4. Limitations associated with androgenesis

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. Although anther culture technique has been used to produce haploids from an array of species, success of the technique cannot be proven in respect of all genotypes of a crop species [11]. Particularly when it comes to the anther culture of Indica rice, the response remains extremely variety or genotype specific [1]. The problem is further aggravated because anther culture response is affected even by the growing season [4].

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 [12]. 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 [13]. Molecular studies carried out on anther culture of cereals such as wheat, barley, and rice have attributed the associated albinism to large-scale deletions and rearrangements in the plastid genome [14].

5. Factors affecting rice anther culture

Investigations on haploid induction through anther culture have been steadily increasing due to its importance as a supplementary breeding strategy. These studies are mainly driven with close monitoring of a number of factors that influence androgenesis in rice as described in detail below.

5.1. Genotype of the donor plants

Response to anther culture by Indica rice varieties is generally poor, and even among those that respond by producing callus, the *in vitro* morphogenic responses are highly genotype-dependent. The recalcitrance associated with the Indica types can be characterized mainly by poor callus induction response, poor regenerability of green plants, and the occurrence of a large proportion of albinos [1]. By comparison, Japonica rice varieties respond much better. Anther culture ability of Japonica varieties, Indica varieties, and their hybrids can be indicated in the following order of Japonica/Japonica > Japonica > Indica/Japonica > Indica/Indica > Indica [15]. Thus, the Japonica varieties have benefitted more from this supplementary breeding approach, and extensive practical applications have been possible. For example, 67,000–159,000 anthers from F1 hybrids of 25–36 rice crosses produced 1500–15,000 (2–10%) green plants per season for selection [16]. Anther culture technique has been used with a Japonica rice variety to purify it and in the process to develop stable new lines that are distinctly different to the parent variety [17]. On the other hand, utilizing anther culture technique for Indica rice breeding has been extremely limited due to its comparatively poor androgenic response [3] with a few exceptions [18]. However, anther culture performance in F_1 hybrids and F_2 plants could be improved when high yielding commercially grown Indica rice varieties were crossed with high anther culture-responsive Japonica varieties [19].

Reference [20] has very convincingly illustrated the extreme variability in anther culture response between Japonica and Indica varieties as 41% for a Japonica variety to 0% for an Indica variety. Not only between the subspecies but also among different varieties from the same subspecies, a considerable variation for callus induction and plant regeneration has been observed. Reference [21] stated that among seven Indica rice varieties on anther culture, callus induction frequencies varied extensively from 3.6 to 51.7%, while green plant regeneration efficiency ranged from 1.6 to 82.9%. Reference [22] reported that out of 18 Indica varieties subjected to anther culture, only five varieties were responsive for pollen callusing, and only

four varieties produced regeneration response. Similarly, reference [23] verified the extremely low androgenic response of Indica varieties as they found only 1 out of 35 Indica varieties exhibited pollen callusing on N_6 medium. The use of optimal media, specifically formulated for each of different genotypes, may help to improve the low response associated with some high valued varieties [13].

5.2. Physiological status of donor plants

Success of the anther culture is greatly influenced by the physiological condition of the anther donor plants. That is mainly because physiology affects the number of viable and healthy pollen grains produced within the anthers, the endogenous levels of hormones that regulate metabolic pathways, and the nutritional status of the anther tissues [4]. During maturation of the anther donor plants, environmental factors such as light intensity, photoperiod, temperature, nutrition, and CO_2 concentration critically affect the growth and development. Also, pest infestations and control measures may have a detrimental effect on microspore development [13].

An improved androgenic response of an Indica rice variety induced by growing donor plants under specific conditions of light and day/night temperature regime was observed by reference [24]. The highest anther response was shown by anthers cultured from donor plants of variety IR43 grown until panicle emergence stage under long days (>12 h), high solar radiation (>18 Mj m^{-2}), and sunshine (>8 h) and day/night temperature (34/24°C), and a declined response was observed when the plants were grown under an environment with low values of the above conditions. They also observed that the plants grown under the field conditions were significantly superior than those grown in controlled conditions such as glasshouse or in pots near the field. Similar observations have also been reported for other cereals, such as maize and wheat. Certain chemicals such as ethereal, when applied on the donor plants, have altered their physiology, thereby enhancing the androgenic response [1]. Further, the anthers collected from the primary tillers were more responsive for anther culture than the anthers from panicles on late tillers [3]. When the anther donor plants were starved of nitrogen, anthers were able to produce much better response in *in vitro* culture compared to those that are given optimum levels of nitrogen fertilizers [2].

5.3. Pollen development stage

The pollen development stage is a critical factor that strongly affects the success of anther culture. Induction of embryogenic calluses cannot be achieved by culturing pollen in any stage of development, and the potential is restricted to specific pollen maturity stages only [4, 25]. For rice, the best responsive stage for embryogenic induction has been reported to be the middle to late uninucleate stage of microspore division, and therefore anthers need to be cultured at these specific stages [26–28]. These are very early stages of microspore development. The highest response shown by these early stages is most likely due to the fact that they are cells which have not yet been committed to gametophytic development and therefore can be forced to become proliferative. The undifferentiated cells in the callus can then assume a new pathway of development leading to sporophyte formation [4].

Although it is stated that the best responsive stages are the middle to late uninucleate microspores, the precise stage of microspores that is best suited for producing a superior anther response can vary from one genotype to another. Therefore, application of the anther culture technique requires detailed examination of pollen before culture to determine its effective development stage [3]. When culturing rice anthers, determining the microspore development stage requires nuclear staining and cytological examination of microspores prior to culture. However, repeating nuclear staining of microspores with each rice panicle, before dissecting anthers for culture, greatly impedes the anther culture process. Therefore, a distinct morphological indicator trait that correlates well with the stage of microspore maturity is commonly used during *in vitro* culture. In rice anther culture, the morphological trait that has been used is the measured distance between the nodes of the last two leaves: the flag leaf, and the penultimate leaf [27, 29]. In some cases the panicle length at the time of harvest has also been used as a visually identifiable guide [30, 31]. The use of a direct cytological marker such as the degree of starch accumulation in microspores has been identified as more accurate than the internode distance and even more convenient than the laborious nuclear staining process to rapidly assess microspore maturity. The most appropriate stage is when pollen grains just begin to accumulate starch which can be simply tested with I_2/KI solution [32].

5.4. 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 [3].

The most commonly used basal media for anther culture are N_6 medium [33], MS medium [34], and B5 medium [35]. Generally, basal N_6 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 morphogenic 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.

5.4.1. 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 latter stages of culture seem to be deleterious for cereals [4]. 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. Maltose has been identified as a superior source of carbohydrate compared to sucrose for androgenesis in cereals. Anther culture efficiency and green plant formation of highly recalcitrant Indica rice varieties could be improved significantly when sucrose was replaced by maltose [1]. Reference [23] reported an inferior anther culture response with sucrose, as only 1 out of 23 Indica rice varieties responded with pollen callusing and green plant production on N_6 medium provided with 146 mM sucrose. 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%. Reference [36] had observed that 20% maltose used for microspore isolation and 9% maltose used for culturing produced a genotype-independent plant regeneration response. 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. The toxic effects of sucrose on androgenesis have been attributed to the sensitivity of microspores to fructose. This also causes depletion of sucrose in the medium with time [1]. Comparatively, long-term availability of maltose in the culture medium has been detected due to the slow rate of hydrolysis.

5.4.2. 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 [3].

The N_6 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 [1]. Reference [37], in which the response of eight Indica rice varieties were studied on different media, found He_2 medium to be more effective than N_6 medium. He_2 medium is derived from the N_6 medium by reducing NH_4^+ concentration to half strength. In Korea, N_6-Y_1 medium which is similar to N_6 except that the $(NH_4)_2SO_4$ concentration is reduced from 3.5 to 1.5 mM has been recommended for Indica-Japonica hybrids [5]. Reference [38] reported that 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}.

Reference [39] 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 [4].

5.4.3. 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 [4].

The growth regulator 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 [24]. 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. Not only the growth regulator combination but also the auxin/cytokinin balance has been found critically important for effective androgenesis. Reference [23] reported that the growth regulator regime of 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. 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 [4].

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 [22]. 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 [9]. 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 [24].

5.4.4. Physical state of the medium

Usually, rice anthers are cultured on solid media. However, reference [23] found increased necrosis of anther tissue when they were cultured on solid media and observed a better callusing response in liquid media. 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, severalfold increment in green plant regeneration comparable with the rates reported for Japonica rice varieties/hybrids could be obtained [29].

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. These have been identified as barriers for the use of liquid media for androgenesis. 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 [3]. 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 [13]. Some have found improved response by embedding anthers in agarose than culturing on semisolid or liquid media [40].

5.4.5. 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. For two Indica rice varieties, Nona

Bokra and Pokkali, callus induction frequencies and plant regeneration responses could be improved when cultures were incubated at alternating temperature regime of 30/20°C (14/10 h) instead of constant incubation at 25°C [5]. 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. Reference [41] reported the effectiveness of culture conditions such as 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). Regeneration phase requires even more specific incubation conditions to achieve success, particularly for green shoot formation. 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. 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 [42].

The composition of the atmosphere in the culture vessel has not been thoroughly studied despite its importance shown with tobacco [4]. Explant density and explant orientation in the culture medium also have been found to be critical in anther culture [2, 4].

5.5. Pretreatments to trigger androgenesis in rice

In many crop varieties including cereals, usually a treatment applied to excised anthers, inflorescences, or anther donor plants prior to culture is important to trigger the sporophytic development deviating from normal pollen development pathway. The type of the effective pretreatment, duration, and the time of application vary with the species or even for different varieties [1, 4]. Reference [43] reviewed the different pretreatments which are in current applications for triggering the anther culture response, and they have been classified into three categories based on their utility as widely used, neglected, and novel. These pretreatments include high temperature and chilling, high humidity, water stress, anaerobic treatment, centrifugation, sucrose and nitrogen starvation, ethanol, γ-irradiation, use of microtubule disruptive agents, electrostimulation, high medium pH, and heavy metal treatment.

5.5.1. Temperature pretreatment

Most frequently used effective method of pretreatment for rice anther culture is the low-temperature application. Harvested rice panicles are subjected to cold shock 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 [1, 3]. Reference [44] 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 [45]. Panicle pretreatments longer than 11 days tend to increase albino production [46]. Reference [37] reported a brief exposure to high temperature (35°C for 10 min) before the cold treatment to enhance callus induction although it adversely affected green plant production.

5.5.2. Osmotic stress

Osmotic shock has been identified as another pretreatment, which can substitute or be used in combination with cold treatment for the induction of androgenesis. Reference [47] have reported the treatment of anthers in 0.4 M mannitol solution to be effective for inducing androgenesis in microspore cultures of Indica and Japonica varieties. Sole mannitol treatment without the cold pretreatment given to anthers promoted androgenesis in anther cultures of variety IR43 from 3 to 33.4% [48]. 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 [43]. 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 [49].

5.5.3. 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 [43]. Reference [39] reported that cold pretreatment could be partially substituted by subjecting microspores for sugar starvation for 3 days during androgenesis of Indica rice. Reference [47] 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 reference [43].

5.5.4. Irradiation

Penetration of irradiation varies with the species and dependent pollen morphology and the thickness of the pollen wall [50]. Reference [51] demonstrated the stimulation of green plant regeneration from rice anther culture with the application of gamma rays at the dose of 20 Gy. 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 [52].

5.6. Ploidy-level determination and doubled haploid production

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 [13]. Occurrence of non-haploids can be due to different malformations. Tissues formed from somatic tissue of anther walls, the fusion of nuclei, endomitosis within the pollen grain, or irregular microspores formed during irregular meiosis lead to the development of plants other than the haploids. Also, when the vegetative and generative nuclei are not separated by cell wall formation, non-haploids could be originated [4].

Some species are associated with a great tendency for spontaneous chromosome doubling. In such cases haploid cells are directly converted to homozygous DH plants. If spontaneous doubling is completely absent or occurs at a low frequency, the haploid plants resulted in cultures need to be converted to dihaploids by some other means such as application of chromosome doubling agents [53]. Among many such chemicals, colchicine is the most widely used anti-microtubule agent *in vivo* and *in vitro*, and oryzalin and trifluralin have also been used.

The ploidy status of regenerated plants can be determined through direct and indirect measures. Direct determinations include mitotic chromosome counts or use of the flow cytometry technique [54]. Initial attempts to ploidy determination relied on karyotypical assessments which was highly time-consuming, laborious, and difficult due to the requirement of skilled operators and was most of the time a failure due to the unavailability of dividing cells. Recently, flow cytometry has hastened the ploidy status analysis. As an alternative to chromosome counting, some other correlated measurements which do not require actively dividing cells have been reported for estimating the ploidy level. These include leaf stomatal density and size. However, these measurements alone cannot be granted sufficiently reliable [54]. When the diploids arisen from anther cultures were analyzed using SSR markers to determine their source of origin, homozygosity has been detected for all in 150 DHs except for one which turned out as a heterozygote [31]. Therefore, microsatellites and other molecular tools such as isozyme analyses and RAPD markers can be utilized to ascertain the homozygosis confirming that the calluses and plantlets have been arisen from gamete itself and not from other somatic tissues.

6. Conclusion

Anther culture technique has been recognized as an efficient alternative to the conventional inbred line development which is usually achieved through a number of inbreeding cycles. A number of critical factors have been addressed for directing the anther culture process for its optimum condition since it can be potentially developed as a supplementary breeding tool for rice.

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Conflict of interest

No other condition or relationship has a potential conflict of interest.

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