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REVIEW

Indica rice anther culture: can the impasse be surpassed?

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Abstract During the past two decades numerous papers have been published on anther culture of rice. These studies clearly indicate that while anther culture is a technique that can be adopted for breeding japonica rice, it being a useful adjunct in indica rice breeding is still some way away. The main reasons why anther culture cannot be utilized for indica rice breeding is analyzed, and aspects that may be manipulated to achieve progress are presented in this review. The two stages of rice anther culture, callus induction and green plant regeneration, are genetically determined traits that show quantitative inheritance. Indica rice is known to have a recalcitrant genetic background that supports these traits poorly. While improvement of the genetic background through recombination or gene transfer remains possible, manipulation of culture media, particularly the nitrogen and carbon sources, has brought about substantial improvements in indica rice anther culture. Adjustments to pre- and post-culture conditions, that include application of various stresses on anthers before and after culture, also have had beneficial effects. The importance of reducing the tissue culture phase to achieve direct embryogenesis is discussed with special reference to improving green plant regeneration potential. The necessity to understand the processes involved in microspore embryogenesis is highlighted in order to support empirical knowledge and achieve a breakthrough in technology. In this regard, rice genome sequence information may be leveraged to elucidate functions of genes involved in microspore embryogenesis.

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Introduction

Anther culture is viewed as a useful technique in plant breeding to rapidly develop homozygous lines and enhance selection efficiency. Regeneration of haploid plants from cultured anthers and their subsequent diploidization, which results in fully homozygous genotypes or doubled haploids, provides an alternative route to the conventional method of inbred-line development that is usually achieved through several cycles of inbreeding. The effectiveness of the technique depends on the efficiency of haploid plant regeneration from microspores contained within the anthers, and the conversion of these haploids to doubled haploids either spontaneously during the tissue culture phase or induced thereafter. With this method, true breeding lines are produced in the immediately succeeding generation. Therefore the technique has immense potential for developing homozygous breeding lines in relatively a short period.

Since the first report on haploid plant production in rice through anther culture by Niizeki and Oono (1968) many early studies have been carried out on various aspects of rice anther culture including pollen ontogeny during culture (Iyer and Raina 1972; Guha et al. 1970). More recently, anther culture technique applied to rice has been greatly improved and its scope expanded to facilitate other biotechnical approaches such as gene transformation technology. Very often gene incorporation results in heterozygosity of the transformed loci. Anther culture has considerable value in shortening the time required to convert the transgenic plants to homozygous breeding lines

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(Baisakh et al. 2001; Otani et al. 2005) or as a technique to be adapted for direct one-step homozygous transgenic plant development (Chen et al. 2006). The potential of anther culture in producing marker-free transgenic rice, a vexed issue with the consumer, has also been recognized (Zhu et al. 2007). However, most developments have occurred in temperate japonica cultivars while success with tropical indica types has remained poor (Kush and Brar 2002; Raina 1997). As a result, anther culture is now used as a supplementary breeding tool in japonica rice (Brar and Kush 2006; Kush and Brar 2002), but the potential of the technique for indica rice breeding is yet to be fully exploited (He et al. 2006) in spite of an initial report of the release of a salt tolerant indica variety through anther culture breeding (Senadhira et al. 2002).

The poor response of indica types to anther culture has been clearly established in many studies. Miah et al. (1985) reported that anther culture response varied from 42% for a japonica cultivar to 0% for an indica cultivar. Sripichitt et al. (2000) testing two indica and one japonica variety found callus induction in the indicas was extremely poor (1.7–4.4%) compared with the japonica variety (17%). A general trend has been observed in anther culture ability of japonica varieties, indica varieties and their hybrids, in the decreasing order of japonica/japonica > japonica > indica/japonica > indica/indica > indica (Guiderdoni et al. 1992; Yan et al. 1996). Even among genotypes of a particular ecotype, japonica or indica, considerable variation in pollen callusing and green plant regeneration has been observed, the genotypic effect being greater among the indicas. For example, among seven indica varieties, callus induction frequencies varied from 3.6 to 51.7% while green plant regeneration efficiency ranged from 1.6 to 82.9% (He et al. 2006). Recent studies on anther culture of indica rice varieties from countries as climatologically diverse as China, Bangladesh, Sri Lanka and Iran, using improved culture media, continue to highlight the genotype specificity of anther culture response within the indica subspecies (He et al. 2006; Shahnewaz and Bari 2004; Ratheika and Silva 2007; Talebi et al. 2007). The recalcitrance displayed by the indica types relates to poor callusing ability, limited morphogenetic potential or regeneration ability of the induced callus, and a higher percent of regenerated plants being albinos (Bishnoi et al. 2000; Roy and Mandal 2005; Talebi et al. 2007). The poor androgenic response of the indica rice thus limits the utilization of this technique as a breeding tool in areas predominantly planted with this ecotype such as the tropical and subtropical regions of Asia.

This review is presented with the objective of discussing the various factors and conditions that impact on indica rice anther culture, and examining critically the aspects that are likely to allow manipulations that may lead to improved success of the technique used with indica rice.

Genetic factors

Quantitative inheritance of anther culture response

Studies on several indica and japonica varieties and their hybrids have shown that callus induction (also termed "anther response") and plant regeneration from the induced callus ("anther culture efficiency") in rice are two independent traits that display quantitative inheritance (Bagheri and Jelodar 2008; He et al. 2006; Miah et al. 1985; Quimio and Zapata 1990; Yan et al. 1996; Zhang and Qifeng 1993). Depending on the study and the plants analyzed, the variation among genotypes to anther culture has been attributed to different genetic sources such as additive genetic effects, non-additive or dominant effects, and sometimes to cytoplasmic effects.

Analyzing the genetical control of anther culture response in two japonica varieties, their F1 and backcross generations, Zhang and Qifeng (1993) reported that the inheritance of callus induction and culture efficiency were influenced by the additive and dominant effects of the genes concerned. Further, they observed that the additive variance component was higher than the dominant component for callus induction whereas dominant variation was more important for regeneration or culture efficiency. Similar observations were made by He et al. (2006) studying a diallel cross involving seven indica parents in which they identified that both additive and non-additive (dominant) effects were important for callus induction while a predominance of non-additive effects were associated with green plant regeneration. In a four-parent diallel cross of two indica and two japonica varieties and their F1s, Miah et al. (1985) also found that additive gene effects predominated in the determination of callus formation ability and that the narrow heritability of the trait was very high in the varieties studied. An incomplete diallel analysis performed using two indica and two Iranian local rice varieties as parents indicated strong additive effects for both the traits, callus induction and green plant regeneration (Bagheri and Jelodar 2008). In a separate study on several indica, japonica varieties and their crosses, the significant contribution of additive genetic effects for callus induction was reiterated although maternal (cytoplasmic) effects were suggested to be important for the determination of green plant regeneration frequency (Yan et al. 1996). A cytoplasmic effect for regeneration of green shoots was also implied for some indica × japonica crosses (Guiderdoni et al. 1992), although results were inconclusive as direct reciprocal crosses had not been evaluated.

These results point to a general consensus of opinion that callus induction from cultured anthers is controlled by additive gene effects to a large extent, with non-additive

effects being less important in determining the anther response. It is also reported that the additive genetic variation component for anther culture traits is higher in japonica varieties than in the indicas (Yan et al. 1996) thus indicating that the genetic determination of the trait is stronger in japonica types. Genetic control of green plant regeneration on the other hand is less well established. Additive, as well as non-additive gene effects, and in some instances cytoplasmic genetic sources have been suggested for the differences in regeneration potential observed among the genotypes. Similar differences of opinion occur over the source of genetic variation in the in vitro response of rice scutellum cultures (Khanna and Raina 1998), and are likely to be the result of the use of different genotypes in experimentation by different authors.

Chromosomal location of the genes and molecular markers

The genes responsible for anther culture response have been recognized to be on specific chromosomal regions in rice. One region in chromosome 1 of rice has been found to control callus formation from microspores and another region in chromosome 10 is understood to be responsible for controlling the balance between albino/green plant regeneration capacities (Yamagishi et al. 1998). He et al. (1998) have identified five quantitative trait loci (QTL) on chromosomes 6, 7, 8, 10 and 12 and two QTL on chromosomes 1 and 9 for callus induction and green plant differentiation respectively, as well as a major QTL for albino plant differentiation on chromosome 9. QTL that influence green plant regeneration have also been mapped on chromosome 3 and 10 and molecular markers that co-segregate with these genes have been identified (Kwon et al. 2002a, b).

Genetic improvement of tissue culture response: potential and limits

Higher additive effects for callus induction and the consequent generation of higher narrow heritability estimates for this trait suggest that considerable genetic gain can be made in the transfer of the trait, callus induction from high responsive cultivars to recalcitrant indica types through hybridization and selection (Yan et al. 1996; Zhang and Qifeng 1993). However, the genetic advance to be achieved in the transfer of the trait, green plant regeneration ability, may not be of comparable magnitude, since the genes controlling the trait show less additive effects and relatively low heritability. It is also the case that these two traits, callus induction and green plant regeneration, are not always correlated positively. Although there are reports of highest callus inducing cultivars showing the best regeneration frequency (Javed et al. 2007; Shahnewaz et al. 2003; Yan et al. 1996), often the genotypes that show high callus induction have displayed poor regeneration ability (He et al. 1998; Talebi et al. 2007). Therefore selection for improvement in callus induction alone may not necessarily improve regeneration ability. Rather, it is important to identify genotypes that show an overall improvement in anther culture efficiency and not merely those that yield callus at high frequency.

A biotechnical approach that would assist in the reliable screening of germplasm for good and bad genotypes for anther culture ability is the development of molecular markers linked to QTL of tissue culture response (Bolibok and Rakcoczy-Trojanowska 2006). Such molecular genetic markers can be leveraged for marker-assisted selection (MAS) strategies that would help to speed up the development of high responding lines with improved anther culture ability in indica rice through introgressive breeding. An isozyme marker, peroxidase, which is closely associated with plant morphogenesis has been used to discriminate between good and bad callus. Anther derived callus from indica rice that had higher levels of the peroxidase enzyme displayed a greater regeneration potential while calli with lower levels of the enzyme gave rise to only albinos or predominantly albinos (Subhadra and Reddy 1998). Recent studies have identified several genes that are specifically expressed in calli with good regeneration ability and some have been cloned. Transformation of a genotype which showed a poor response in tissue culture with a gene that putatively encodes glucose dehydrogenase has greatly enhanced the differentiation rate of calli (Ozawa et al. 2003).

Thus, a genetic approach to improving plant regeneration from anther-derived callus in indica rice can be through sexual hybridization followed by selection for QTL that are favorable for green plant regeneration, perhaps facilitated by the MAS process. It may also be feasible to use a more direct approach through transformation technology when individual genes impacting on different aspects of anther culture are identified and cloned. Irrespective of the method used, it is vitally important that such incorporation of genes should be recoverable in commercially advantageous rice varieties, since the usefulness of plant varieties that are anther culture-endowed would be largely governed by their agronomic features.

The complexities of genetic control of in vitro response, especially green plant regeneration potential, may limit the possibilities of developing high responding genotypes through breeding. However, the existence of a large non-genetic component of variation for regeneration from anther-derived callus suggests that there is still sufficient scope for improving culture efficiency through manipulation of these non-genetic factors that include the culture media compositions and pre- and post-culture conditions.

Culture media

Anther culture in rice is essentially a two-step process of initial development of callus and subsequent regeneration of green plants from embryogenic callus. The nutrient requirements of the two processes vary and therefore are facilitated on different culture media. Since callus induction potential is largely determined by the genes, manipulation of non-genetic factors such as the culture medium may not produce very significant levels of improvement in anther response in highly recalcitrant genotypes. Nevertheless it must be noted that the choice of medium has made a difference in the rate of success in callus induction ability of japonica and indica genotypes. The most widely used medium for inducing higher anther response in japonica cultivars is the N6 medium (Chu 1978) although the basal nutrients of this medium are not optimum for anther culture of indica rice (Lentini et al. 1995; Raina et al. 1989). Potato-2 medium (Chuang et al. 1978) developed initially for wheat anther culture was adopted for rice, with a higher level of success than N6, especially in the anther culture of F1 hybrids from interspecific crosses, *O. sativa* × *O. rufipogon* (Rout et al. 1989; Rout and Sarma 1991). However, subsequent developments have focused mostly on affecting modifications to the two basic media, N6 and MS (Murashige and Skoog 1962), for improving anther culture efficiency in indica rice.

Callus induction media for indica varieties

Several modifications to the basal N6 medium have been tested for their usefulness for anther culture of indica rice. Formulations such as MSN, SK-1, He 2, RZ have been used in which the components modified included the nitrogen level and source, carbon level and source, addition or withdrawal of undefined substances, as well as changes to vitamins and their concentrations (Lentini et al. 1995; Raina et al. 1989; Raina and Zapata 1997).

Nitrogen source

Inorganic nitrogen is usually supplied in the form of nitrate and/or ammonium ions in tissue culture media. The ratio of NO_3^-:NH_4^+ has been observed to be an important determinant for the success of anther culture as well as for the in vitro induction of embryo callus in indica rice (Grimes and Hodges 1990). The N6 medium used successfully with japonica varieties contains both KNO_3 (28 mM) and $(NH_4)_2SO_4$ (3.5 mM) as primary sources of nitrogen. Media derived from N6 that have been used frequently for indica rice anther culture are MSN and SK-1 or others that are slight variants of these. MSN medium which is a blend of MS (Murashige and Skoog 1962) and N6 media, has the N6 nitrogen background while SK-1 has inorganic nitrogen provided only as nitrate ions and without ammonium ions. A comparative study proved that SK-1 was overall a better medium for indica rice anther culture because green plant regeneration was higher from callus induced on this medium than from callus induced on MSN (on average 200–400% higher regeneration of green plants), even though MSN medium was the more effective for callus induction, with some genotypes yielding more than twice the amount of callus from anthers cultured on MSN (Raina et al. 1989). Further investigations by Raina and Zapata (1997) found that nitrogen supplied only in the form of nitrate or ammonium ions in the medium was less beneficial for induction of morphogenic calli from cultured anthers than a combination of both at appropriate concentrations. On this basis a revised medium that was introduced, RZ, in which a lowered $(NH_4)_2SO_4$ (1.75 mM) and an increased KNO_3 (31 mM) concentration used to that in N6, produced better anther response and green plant regeneration ability of anther-derived callus in indica rice (Raina and Zapata 1997). Anther culture on He2 medium and a modified SK-1 medium which had NH_4^+ levels reduced to half that in N6 further demonstrated the beneficial effects of lowered NH_4^+ levels on callus induction and subsequent regeneration of green plants from induced callus in indica rice (Lentini et al. 1995). Media with lower ammonium nitrogen have also been found to be more suitable for wheat and barley anther cultures (Mordhorst and Lorz 1993). Organic nitrogen supplements such as casein hydrolysate added to the medium have not been particularly beneficial for positive anther response (Lentini et al. 1995; Raina and Zapata 1997) although a few reports suggest otherwise (Roy and Mandal 2005). On the other hand amino acids such as glutamine and alanin have proved to be useful for callus formation and green plant regeneration from microspore culture in indica rice varieties (Ogawa et al. 1995).

Carbon source

The effect of the carbon source on callus induction in rice anther culture has also been studied extensively. In early studies, media designed for rice anther culture used sucrose as the standard carbon source. However, the type of carbon source in the medium has been shown to affect significantly, the anther response in rice like in other cereals such as barley and wheat (Finnie et al. 1989; Kuhlmann and Foroughi-Wehr 1989). The superiority of maltose over sucrose as the carbon source in rice anther culture was aptly demonstrated by Lentini et al. (1995). Replacing sucrose (146 mM) with maltose (146 mM) in the callus induction medium had a significant positive effect on anther response in both indica and japonica types, with a

greater effect on indicas. Not only did maltose improve callus induction frequencies, callus induced on media containing maltose was better able to regenerate plants and for some genotypes, maltose also raised the ratio of green plants to albinos (Lentini et al. 1995). The beneficial effect of maltose in improving anther culture efficiency has been further substantiated by others (Bishnoi et al. 2000; Pande and Bhojwani 1999; Trejo-Tapia et al. 2002). Bishnoi et al. (2000) in their work with indica parents and indica × basmathi hybrids realized callus induction frequencies of up to 34% in parents and up to 78% (as high as those obtained for japonica varieties) in some hybrids using the modified RZ medium that contained maltose (4% w/v) instead of sucrose. Green plant regeneration frequency of anther calli initiated on this medium was also reported to be quite high, and in some genotypes 51%. While improvements in anther response and culture efficiency in the above studies have been obtained with maltose incorporated into the callus induction medium, more recently it has been shown that maltose added also to the regeneration medium enhanced green plant regeneration in two indica varieties, Nona Bokra and Pokkali (Javed et al. 2007).

Sugars incorporated into tissue culture media have a dual role, as a carbon source and as an osmotic regulator. Media incorporated with different sugars, such as glucose, fructose, mannitol or a combination of these at concentrations that produce equivalent osmotic effects in the medium, have proven to be much less effective than maltose in inducing anther culture response. This has prompted the reasoning that the type of sugar is more important in its role as the carbohydrate source than in regulating the medium osmotic potential for successful anther culture (Bishnoi et al. 2000; Lentini et al. 1995). Maltose in the anther culture medium is degraded more slowly than sucrose and yields only glucose upon hydrolysis. Sucrose on the other hand is metabolized very rapidly into glucose and fructose. Fructose is known to have a detrimental effect on embryoid production in wheat anther culture (Last and Brettell 1990; Navarro-Alvarez et al. 1994) and may also be the reason why maltose supports androgenesis better in rice than sucrose.

Ethylene inhibitors

Lentini et al. (1995) reported that incorporation of $AgNO_3$ (10 mg/L) in the callus induction medium not only promoted anther response in indica rice but also the regeneration of green plants from the induced callus. However, callus transferred onto regeneration medium with $AgNO_3$ did not show a significant improvement in the frequency of green plants produced, suggesting that $AgNO_3$ has a greater effect on the first stage of induction of morphogenic callus more than the subsequent regeneration of green plants from this callus. Enhanced callusing may be a consequence of $AgNO_3$ blocking the inhibitory effects of endogenously produced ethylene from excised anthers (Ghamemi et al. 1994). Omission of sucrose (and replacement with maltose) in the callus induction medium may also influence this process indirectly because the in vitro generation of ethylene in excised tissue is promoted by sucrose (Zhou et al. 1991).

The role of polyamines in anther culture response in rice has also been investigated and it has been suggested that its effect is through inhibition of ethylene synthesis (Dewi and Purwoko 2008). Addition of the polyamine putrescine (10^{-3} M), into the N6 callus induction medium delayed and reduced the senesce of cultured anthers in both japonica and indica varieties. The effect of putrescine application on enhancement of callus induction and green plant regeneration was greater for the indica variety "Krowal" compared to japonica variety "Taipei 309", even though the absolute number of plants regenerated remained higher in the latter variety. Further, the presence of putrecine in the medium was shown to increase the ratio of green/albino plant regeneration frequency for the indica variety from 0 to 3.3%, and the number of green plants per anthers inoculated from 0 to 12.7%, while in the japonica variety the two ratios increased from 2.1 to 3.3% and 2.5 to 36.1%, respectively.

The low anther culture responsiveness of indica genotypes has been connected with the early senescence of anthers introduced into culture. The senescence is likely accelerated with the in vitro generation of ethylene by excised anthers and accumulation of the gas in sealed culture vessels. This appears to be more of a problem for indicas than for japonica varieties. Thus the use of polyamines or $AgNO_3$ as inhibitors of ethylene biosynthesis is likely to be advantageous in anther culture of rice, especially for the indica varieties.

Plant growth regulators

The growth regulator commonly used in the callus induction medium for rice anther culture has been 2,4-dichlorophenoxyacetic acid (2,4-D) at fairly high concentrations (2 mg/L). This has produced relatively high rates of callus induction, of up to 15%, in some genotypes (Raina and Zapata 1997). The effect of several other auxins such as αNaphthaleneacetic acid (NAA), Phenylacetic acid (PAA), picloram and dicamba have been tested either alone or in combination with 2,4-D for their usefulness for in vitro anther response (Lentini et al. 1995). These studies showed that the mean callus induction frequencies could be raised up to 28.7% in indica and japonica genotypes by coupling of 2,4-D (2 mg/L) with picloram (0.07 mg/L), whereas 2,4-D (2 mg/L) combined with NAA (2 mg/L), or NAA (2 mg/

L) alone was much less effective and yielded callus induction frequencies of 18.5 and 0.1% respectively. The effect of dicamba was similar to 2,4-D with respect to callus induction. However, plant regeneration from callus induced on dicamba medium was only one-third of that observed from callus derived on medium containing 2,4-D. Incorporation of PAA in the callus induction medium was detrimental for anther response and inhibited callus induction frequencies by about 10 times even in the most responsive genotypes, and plant regeneration by as much as 20 times (Lentini et al. 1995).

Considering the auxin/cytokinin balance, the growth regulator combination that was favorable for enhanced anther response in a large number of genotypes proved to be 2,4-D (2 mg/L), picloram (0.07 mg/L) and kinetin (0.5 mg/L; Lentini et al. 1995). Bishnoi et al. (2000) used the same growth regulator regime, but with further addition of NAA (2 mg/L), in the callus induction medium to produce similar levels of success. Even though 2,4-D has proven to be a potent auxin for callus induction from cultured anthers, it has been recognized that the regeneration ability of callus induced under high 2,4-D levels is poor, especially for indica rice, in comparison to callus induced on medium with lower 2,4-D levels (Raina and Zapata 1997). This has been borne out in more recent studies where reduction of the level of 2,4-D to 0.5 mg/L (and omission of picloram altogether) in the callus induction medium appeared eventually to be the most effective for anther response, even though confounding effects from modifications made to various other culture media components did not allow a clear 'cause and effect' relationship to be established between growth regulators and anther response (Bishnoi et al. 2000). A lower level of 2,4-D (0.5 mg/L) in combination with the milder auxin NAA (2.5 mg/L), and kinetin (0.5 mg/L) have been used effectively to induce callus from several indica varieties (Shahnewaz et al. 2003; Shahnewaz and Bari 2004) and have been observed to be desirous for the overall efficiency of green plant regeneration from the induced callus (Raina 1997). A combination of the same three growth regulators in somewhat similar proportions (2,4-D 1 mg/L, NAA 2.5 mg/L, and kinetin 0.5 mg/L) have had a positive influence on callus induction from cultured anthers of the interspecific hybrid, *O. sativa* × *O. rufipogon,* while the regeneration frequency of calli produced in the presence of both auxins was also reported to be higher (Rout and Sarma 1991).

Growth regulators used in the regeneration medium and their effects on green plant production has been analyzed to a lesser extent than in the case of callus induction. Bishnoi et al. (2000) reported that medium supplemented with NAA (0.5 mg/L), BAP (1.0 mg/L) and kinetin (1.0 mg/L) has adequately supported green plant regeneration from sub-cultured callus.

Liquid or solid media?

Rice anther culture commonly uses solid media. However, Lentini et al. (1995) favored liquid media for callus induction. They found that agar solidified media increased anther necrosis. It has been suggested that liquid culture systems may provide microspores and calli with greater access to nutrients and hormones while dispersing more rapidly the toxic substances released from dying or dead anthers. The tendency for the rice anthers to sink in liquid media and rapidly lose viability has been a major reason for avoidance of liquid culture conditions by many (Raina 1997). Efforts to keep anthers afloat and viable in liquid culture, by adding substances such as Ficoll that increases buoyancy, has been reasonably successful. Others have found that embedding anthers in agarose was better than culturing on semi-solid or liquid media (Gill et al. 2000).

Callus induction medium and its effect on regeneration ability of callus

The main objective of anther culture is to increase culture efficiency by increasing the frequency of green plant regeneration from cultured anthers or microspores. It may be argued that this could be achieved by parallel improvement of the two phases of the anther culture process, induction and regeneration. However, many studies have demonstrated that the induction medium plays a pivotal role in determining the success of green plant regeneration. Therefore it is not surprising that efforts have been directed largely towards optimizing induction media through manipulation of tissue culture components and conditions, with less attention being paid towards possible manipulations to regeneration media. However, it is crucial to ensure that the callus induced is largely embryogenic with good regeneration potential, so that when transferred to regeneration media are capable of green plant production. Thus, the emphasis should be on identifying media requirements primarily for the production of embryogenic callus, and then capitalize on the 'quality aspect' of the callus induced to achieve the targeted regeneration frequencies rather than indulge in media that give rise to prolific callusing that eventually fail to regenerate.

Genotype × medium interactions

Interactive effects of genotypes with culture media have been observed in callus induction from cultured anthers in rice. In 7 Iranian rice varieties tested on 3 callus induction media, Talebi et al. (2007) reported specific media requirements for anther response by different genotypes. The best two varieties, Domsiah and Anbarbo, showed

strong genotype × medium interactions for callus induction. In the variety Domsiah, the maximum callus induction frequency of 34.1% was observed on medium designated as Fj, while the induction potential fell to a remarkable low of 6.6% on a medium identified as L8. Conversely, in the variety Anbarbo, callusing frequency was reduced to 17.7% on Fj medium from a high of 32.5% in L8. Both media were devoid of 'ammonium' nitrogen, but had supplementary nitrogen sources in the form of glycine in Fj and lactalbumin in L8. Similar genotype-specific culture media requirements in rice anther culture have been discussed by others (Javed et al. 2007). Genotype × medium interactions have been observed in the culture of somatic tissues in rice too. Significant interaction effects of genotype × callus induction medium, genotype × plant regeneration medium, as well as interactions between genotype, callus induction medium and regeneration medium have been detected for scutellum cultures of 3 indica varieties on 8 media formulations (Khanna and Raina 1998).

These results appear to underscore an inescapable reality that while media manipulations would bring about appreciable levels of improvement in anther culture ability of a wide range of indica rice germplasm, culture media requirements may remain specific for certain genotypes and optimization still may have to be done on individual genotype basis.

Donor plant effect

Physiology of the donor plant has also been identified as an important contributory factor for the success of rice anther culture, with seasonal variations observed in the anther response (Datta 2005; Raina 1997). For example, long days (>12 h), intense solar radiation (>18 MJ/m^2) and higher day/night temperature regime of 34/24°C at panicle emergence produced the best anther culture efficiency in the indica cv, IR 43. It was also noted that anthers collected from the primary tillers were more responsive in comparison to panicles developed on late tillers. Anthers of panicles collected from field grown plants have been decidedly better in their anther culture response compared to anthers collected from pot plants placed in the green house or near the field (Raina 1997; Veeraraghavan 2007).

Pollen development stage

The ability of pollen to redirect its development pathway from one of gametogenesis to that of embryogenesis depends on the stage of maturity of the pollen grains at the time of culture. For rice, the most suitable stage of pollen development has been described as the late uni-nucleate to early bi-nucleate stage as well as early to mid uni-nucleate stage (Jahne and Lorz 1995). This suggests that the exact pollen stage that is capable of producing the best anther response, although within this range, may change from one genotype to another. Therefore pre-examination of pollen to determine its development stage will be imperative to optimize anther culture response before embarking on a large-scale anther culture program. An easily observable morphological trait of the plant that shows good correlation with the pollen development stage is used as a guide to identify the required stage of pollen. Usually the distance between the collar of the flag leaf and ligule of the penultimate leaf of the tiller serves as a reliable guide to anther maturity (Bishnoi et al. 2000).

Explant pre-treatment

Several different types of stresses applied on anthers or isolated microspores prior to culture have shown to improve androgenesis in rice as in other cereals (Shariatpanahi et al. 2006). The more notable conditions among the stresses that promote androgenesis are the temperature pre-treatment, commonly administered as a cold-shock rather than a high temperature treatment in rice, osmotic shock, and sugar starvation (Bhojwani and Razdan 1996). The duration and time of application of the stress may vary with the type of stress and variety of rice (Datta 2005).

Cold pre-treatment

The most commonly used is the low temperature pre-treatment in which the harvested panicles are subjected to cold shock. Cold pre-treatment of anthers lasting several days is believed to delay the degeneration of microspores and anther wall tissue in rice (Raina 1997). The optimal temperature and treatment duration may vary with the variety. Panicles have been subjected to a range of temperatures from 4 to 12°C for 07–30 days in different experiments, although prolonged cold treatment of over 11 days have induced development of albinos during the regeneration phase (Gupta and Borthakur 1987). In general, temperatures from 8 to 10°C for 8 days have been recommended to be optimal for many varieties of rice (Zapata-Arias 2003). The role of temperature in androgenic induction has been discussed by Touraev et al. (1997).

Osmotic stress pre-treatment

Osmotic stress has been sometimes used to supplement or replace cold pre-treatment in rice anther culture. Raina and

Irfan (1998) have reported that treatment of anthers in 0.4 M mannitol was useful to induce androgenesis in microspore culture of indica and japonica cultivars. In the absence of cold treatment, mannitol treatment promoted androgenesis in anther culture of indica cultivar IR 43 from 3 to 33%, although no promotion in anther culture ability was observed when the mannitol treatment was combined with the cold treatment (Pande 1997).

Sugar starvation

Sugar starvation is effective in inducing embryogenesis, particularly in isolated microspores. Ogawa et al. (1995) reported that sugar starvation of microspores for 3 days, could substitute to some extent, the cold treatment for the induction of androgenesis in microspore cultures of indica rice. Sugar starvation of anthers of indica rice variety IR 43 for 2 days at the beginning of culture caused a 12-fold increase in the androgenic response in this variety. However, cold treatment was superior to sugar starvation. Sugar starvation has been found to be critical to promote high frequency embryogenesis and plantlet regeneration from microspores isolated from anthers of indica and japonica rice by Raina and Irfan (1998) also.

Culture conditions

Incubation temperatures

Culture temperature is an important factor in the anther culture of japonica rice (Okamoto et al. 2001). Yet not much attention has been directed towards attempting to improve regeneration efficiency by manipulating incubation temperatures in indica rice anther culture. For two indica rice varieties Nona Bokra and Pokkali, callus induction frequencies could be improved by 1.2 and 1.7 fold respectively, and green shoot productivity from 0 to 0.1% and 0.5 to 4.3% respectively (8.6 fold increase for Pokkali variety) when cultures were incubated at alternating temperature regime of 30/20°C (14/10 h) instead of continuous incubation at 25°C (Javed et al. 2007). Therefore it is worthwhile to investigate further, the effect of incubation temperature on anther culture efficiency of indica genotypes.

Osmotic stress

A detailed study on stimulatory effects of water/osmotic stress on shoot regeneration from scutellum-derived callus of indica rice has been reported (Jain et al. 1996). The study examined the influence of increase in agarose concentrations and mannitol levels in the regeneration medium (that directly affected the water content of the callus), as well as partial desiccation of callus before transfer to the regeneration medium, on the regeneration potential of anther-derived callus. Agarose concentration increased from 0.5 to 1% (w/v) produced over 8 fold increase in shoot regeneration in the indica rice varieties IR 43 and Pusa basmati 1. In comparison, shoot regeneration was only slightly enhanced when callus was kept for 4 weeks on regeneration medium containing mannitol at 0.1 M, while higher levels of mannitol (0.2 and 0.4 M) resulted in lowering of the regeneration response. However, upon movement of this callus to mannitol-free medium after 4 weeks, shoot regeneration was clearly enhanced in all treatments. Dehydration of callus in an empty sterile Petri dish (devoid of medium) and subsequent transfer to regeneration medium enhanced shoot regeneration by threefold in variety IR 43 and 2.3 fold in Pusa basmati 1.

These results seem to indicate that a mild osmotic stress is beneficial for indica rice regeneration from scutellum-derived callus. It is possible that similar stimulatory effects can be expected in anther-derived callus also. All of these treatments have been observed to reduce the water content in the callus making the cultures more compact and embryogeneic thus improving overall regeneration.

Albino plant production

The occurrence of a large proportion of albinos among the regenerated plants following anther culture is the most frustrating feature of androgenesis and remains a formidable obstacle for application to rice breeding. The frequency of albinos may vary from 5 to 100% (Talebi et al. 2007). Indica rice cultivars are more prone to this problem than japonica rice varieties. As discussed previously, several factors including pre-treatment conditions and culture medium, affect the frequency of albinos. The literature on androgenesis in cereals suggests that albinism can be considerably reduced by shortening the culture period (Karim et al. 1991; Asaduzzaman et al. 2003). Manipulation of in vivo and in vitro conditions that will circumvent the callus induction process and lead to direct pollen embryogenesis, may be the means to achieve this end. It has also been suggested that the stressful in vitro conditions in the tissue cultures make the plant cells fight their own plastids with antibiotic-like compounds (Torp and Andersen 2009). The presence of large-scale deletions in some plastid genomes of the albino haploid plants derived from anther culture of japonica × indica hybrids and absence of such deletions in green regenerants (Yamagishi 2002), appear to suggest a role for the plastid genome in the determination of the albino phenotype. Even though genetically determined, the trait is amenable for

manipulation, at least to some extent, by culture conditions. For instance, combination of starvation and cold stresses applied simultaneously for shorter periods (3–4 days) have increased microspore survival and reduced the frequency of albino production compared to only prolonged cold-pretreatments (Torp and Andersen 2009).

Conclusion

Genotype no doubt is a deciding factor in achieving success in anther culture response, and the genetic makeup of indica subspecies clearly does not lend itself towards effortless in vitro anther culture. However, components of tissue culture media are also important, and have been demonstrated to have a crucial role in coaxing an in vitro response from cultured anthers of otherwise recalcitrant genotypes. Although a broad set of conditions that apply across many genotypes that boost anther response have been identified and can be specified for indica rice, each genotype will still have individual requirements and these will have to be considered on a case by case basis.

Advances made to the anther culture technique in rice have been largely based on empirical studies that involved manipulation of media components and culture conditions. While continuing work in this direction, it will be also necessary to have a better understanding of the processes involved in microspore embryogenesis to achieve a real breakthrough in technology. In this era of genomics it may be possible to identify genes that are involved in reprogramming the microspores leading to embryogenesis. Understanding of fundamental processes and focus toward technical detail will likely lift the anther culture procedure in indica rice out of its present impasse.

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