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High throughput anther culture response in an upland rice cross 'Khandagiri x Dular'

S.K. Tripathy*

Department of Agricultural Biotechnology, College of Agriculture, Odisha University of Agriculture & Technology, Bhubaneswar, India

***Corresponding Author Email :** swapankumartripathy@gmail.com

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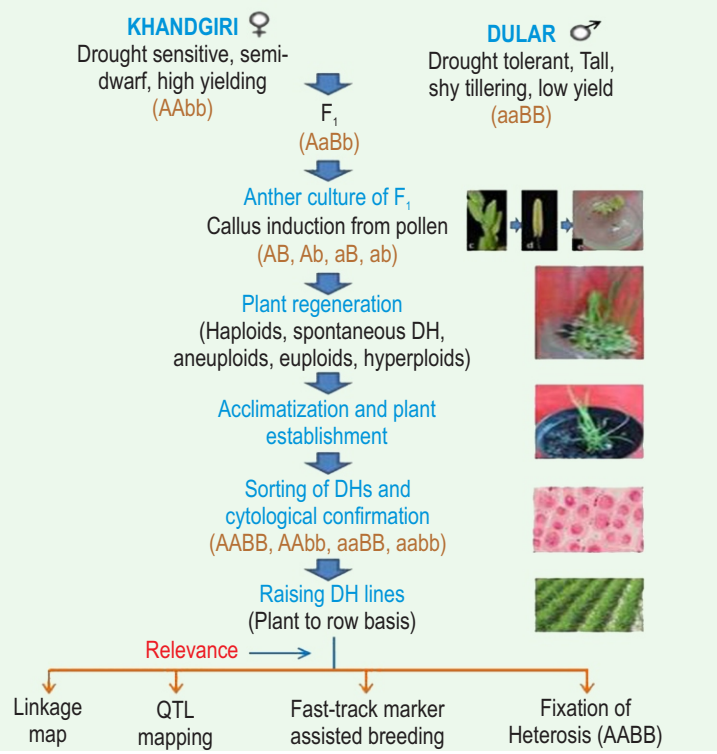
Abstract

Aim: The experiment was conducted to maximize anther culture response in an upland indica rice cross amenable for drought tolerance breeding.

Methodology: The cold pre-treated anthers of a cross Khandagiri (drought sensitive) x Dular (drought tolerant) were cultured on N6, SK3, MS and CIM media at varying concentration of hormones (2,4-D, Kn and NAA) to assess callusing response. Embryogenic calli were placed on regeneration medium (RM) with varying concentration of Kn, BAP and NAA for plant regeneration. The plantlets were acclimatized in half-strength MS basal liquid medium for one week before transferring to pot mixture (peat moss: perlite 2:1, v/v) for plant establishment in glasshouse under partial shade. Finally, the plantlets were checked for doubled haploid status by cytological study of root tips.

Results: Anther culture response was found to be media and genotype-specific. F_1-progenies responded better than either of the parents. A modified MS callus induction medium (CIM) with 1.5mg/l 2,4-D + 0.5mg/l Kn resulted impressively higher callusing response (30.2%) with nodular calli than SK3>MS>N6. High frequency (12.8%) of albino-free green plant regeneration with well developed rooting was achieved in RM medium (a modified MS) containing 2mg/l BAP and 0.5mg/l NAA. A total 129 doubled haploid (DH) plants (each with 12 pairs of chromosomes) were recovered which maintained normal growth, set seeds and resulted in true breeding DH lines.

Interpretation: The high throughput regeneration system is amenable for doubled haploid production from indica crosses. Besides, the present doubled haploid stock can serve as an ideal mapping population and as such targeted for marker aided selection for early development of drought tolerant rice varieties.



Key words: Anther culture response, Doubled haploids, Indica cross, Upland rice

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Introduction

Rice (*Oryza sativa* L., 2n = 24, family Poaceae), is one of the world's most important staple cereal food crop that feeds over half of the global population. It is grown in 120 countries covering 162.06 million hectares with a production of 497.69 million metric tons milled rice in 2019-20 (Shahbandeh, 2021). China and India contribute 50% of global rice production. Asia alone meets 90% of global milled rice requirement. In India, rice is cultivated on 44.00 million hectares area with a production of 103.00 million tons and productivity of 2.34 t ha^{-1} (Shivani *et al.,* 2019). India's current status of rice production needs to be increased by 18.83% (122.4 million metric tons) (Kumar *et al.,* 2016) to feed estimated 1.5 billion people by 2030. Genetic improvement of rice in India and many other rice growing countries has already achieved yield plateau for medium land irrigated rice ecosystems, but areas pertaining to rainfed rice ecosystems particularly high land situations continue to have low productivity owing to scanty and erratic rainfall distribution.

About 8% of the global rice-growing area is in uplands (Saito *et al.,* 2018) and nearly two-thirds of it is in Asia. In India, Odisha in particular has vast stretches of marginal uplands which mostly remain fallow even during rainy season. These areas are often inhabited by socially disadvantaged ethnic minorities for whom food security remains a daily battle. Therefore, there is a need to reorient breeding strategy to develop suitable varieties for the above areas to feed the rural poor. Rice is a highly self-pollinated crop. Development and selection of desired pure breeding lines normally need 8-9 cycles of selfing in conventional breeding. In contrast, the anther culture has proved to be a suitable technique for early fixation of homozygosity (Naik *et al.,* 2017). Consequently, each doubled haploid line derived from the anther culture of F_1 hybrids would bypass the inbreeding process (Germana, 2011) and produce a new true breeding line with unique gene combination (Maria *et al.,* 2006) resulting in increased selection response (Dwivedi *et al.,* 2015).

Anther culture in rice was first reported by Niizeki and Oono (1968) and it appears to be a suitable alternative for genetic improvement in rice. But, recalcitrance nature of indica rice to androgenesis (Xa and Lang, 2011) is the major hindrance to double haploid breeding. Currently, drastic changes in environments (high temperature, heat, cold, salinity and flood) necessitate fast track crop breeding to sustain food production. Drought is the major limitation that negatively affect grain yield in rainfed ecosystems (Mahender *et al.,* 2019) and for this doubled haploid breeding could be a fitting adjunct to conventional breeding. Odisha is the genetic paradise of rice germplasm resources. Among available local land races, cv. 'Dular' is known to have higher level of drought tolerance, while 'Khandagiri (drought sensitive)' continues to be a widely adaptable popular high yielding upland rice variety over different agro-climatic conditions of Odisha. Shamsudin *et al.* (2016) also reported inherent drought tolerance in several traditionally moderate tall landraces such as Azucena, Dular, Rayada and Nagina 22, however, the short duration upland rice varieties so far developed are rarely adaptable to pre-and post-monsoon drought stress. Therefore, the present investigation was planned to develop an anther culture derived rapid regeneration system in an upland indica rice cross "Khandagiri (high yielding and drought sensitive) and Dular (drought tolerant tall land race)" amenable for doubled haploid breeding for drought tolerance.

Materials and Methods

The present study was carried out in the Department of Agricultural Biotechnology, College of Agriculture, OUAT, Bhubaneswar (India) during 2018-19 as a part of ongoing rice breeding programme.

Plant materials: F_1 progenies of an upland rice cross combination "cv. Khandagiri (as female parent) x cv. Dular (as male parent)" were purposefully made as the source of anthers for *in-vitro* androgenesis. Prior to collection of boots, all putative F_1 plants were tested for hybridity using the chromosome 1 major drought stress related QTL specific SSR marker RM 8085, Chr.1 (F: 5'- TGCGTTTCGATTTCTTTTTA 3' and R: 5' GGAAAGTTGTGTTCTTTGGC 3') (Arvind Kumar *et al.,* 2011) alongside both the parents.

Preparation of plant materials for anther culture: Boots from primary tillers of both parent varieties and F_1 (first filial generation) plants were collected around 8.00 a.m. when the anthers occupy 1/3 to 1/2 of the spikelet length or when the auricle distance between the flag leaf and penultimate leaf reached 5-8 cm. The boots were wrapped with tissue paper followed by alluminium foil and placed in the refrigerator at 4°C for one week in dark. After cold pre-treatment, the spikelets from middle portion (Alejar *et al.,* 1995) of the panicles were disinfected with aqueous mercuric chloride (0.1%, w/v) for 1 min under aseptic condition followed by washing (x 5) with sterile distilled water and excess water was soaked with sterile blotting paper. As described by Lentini *et al.* (1995), individual spikelets were held at tip portion by forcep and cut slantly at basal 3/4th position (just below the anther) to detach anther filaments and then to release anthers over the media.

Media used for anther culture: Three standard basal media, *i.e.,* N6 (Chu *et al.,* 1978), SK3 (modified N6: Chen *et al.,* 1978) and MS (Murashige and Skoog, 1962) and a formulated callus induction medium (CIM: a modification of MS) (Table 1) with different hormone recipes (2,4-D: 2,4-Dicchloro-phenoxyacetic acid, Kn: Kinetin/6-Furfuryladenopurine and NAA: α-Naphthalene acetic acid) were used for callus induction. Besides, a regeneration medium (RM) with Kn, BAP (6-benzyl amino purine) and NAA at different concentration was also prepared as a modification of MS medium to suit plantlet regeneration from anther derived calli.

The RM medium was formulated with increased amount of Fe (iron), less of $CaCl_2$ and inclusion of $(NH_4)_2$ SO_4 (232 mg l^{-1}), Glutamine (500 mg l^{-1}l), Tryptophan (100 mg l^{-1}), Cysteine (40 mg l^{-1}),

Casein hydrolysate (500 mg l^{-1}), adenosine sulphate (200 mg l^{-1}), proline (500 mg l^{-1}) along with double the amount of sucrose (6%) as compared to standard MS basal media. The pH of each medium was adjusted to 5.7-5.8, solidified with agar (0.6-0.8%) and sterilized by autoclave at 15 psi for 15 min.

In-vitro culture process and culture conditions: Anthers from selected spikelets of the same panicle were plated on the callus induction medium under laminar air flow cabinet just by tapping the forcep (holding spikelet at top) at the brink of the culture vessel. The culture vessels were incubated for 3-8 weeks under dark in the culture room at 25+1°C and 65% relative humidity (RH) for callus induction (Tripathy, 2020). Calli of approximately 0.5 cm diameter were transferred to regeneration medium (RM medium) and incubated in the culture room under light intensity of 1000 lux at photoperiod 16/8 hrs (light/dark) to facilitate regeneration. Some of the treatments developed only albinos or green plantlets or both. Well developed green shoots were transferred to MS medium without phytohormones for root initiation.

Plant establishment: The plantlets regenerated were initially transferred to half-strength MS basal liquid medium for one week before transfer to pots filled with mixture of peat moss: perlite 2:1 (v/v). Thereafter, plantlets were successfully acclimatized and cultivated in green house under partial shade.

Cytological studies: Spikelets having microspores at late uninucleate to early binucleate stage are in vogue suitable for anther culture (Mishra *et al.,* 2011). For this, cytological stage of central spikelets of a few collected boots were fixed in acetic acid: ethanol (1:3) for 24 hrs followed by staining of pollen grains with acetocarmine (1%) to check the microspore developmental stage under compound microscope. For chromosome counting of doubled haploids, root tips were pre-treated with 0.002 M 8-hydroxyquinoline for 2 hr at 25°C followed by fixing the material with Carnoy's fixative (6:3:1 absolute ethanol: chloroform: glacial acetic acid) for two days and then preserving in 70% ethanol in refrigerator until mitotic slide preparation. The root tips were digested with enzymatic mixture of 6% cellulase and 2% pectinase (prepared in 0.01 M citrate buffer) in an Eppendorf tube at 37°C for 1 hr using water bath followed by staining with 1% acetocarmine on glass slide; and chromosomes at pro-metaphase stage were counted under a phase contrast microscope.

Statistical analysis: Each treatment comprising specific hormone recipe for callus induction as well as plantlet regeneration was laid out in CRD (Complete Randomized Design) with three replicates (each replicate comprised 100 anther). Data were recorded for percentage of anthers induced calli (expressed as CIF: callus induction frequency) as well as percentage of anthers that regenerated to plantlets in culture following transfer of calli induced in CIM + 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn to regeneration medium. Further, the morphogenetic potential of anther derived calli was assessed as number of plantlets /responsive callus. The data set for callus induction was subjected to analysis of variance following factorial CRD (Dafaallah, 2019). Further, the data set for CIM medium having higher response to anther culture, and the regeneration medium specially designed for plant regeneration were subjected to Duncan's Multiple Range Test (Duncan, 1955) to implicate efficacy of the protocol.

Results and Discussion

With the rapid environmental adversities, there is indeed a demand of climate resilient drought tolerant upland rice varieties. Double haploid breeding can be an appropriate way to achieve the task.

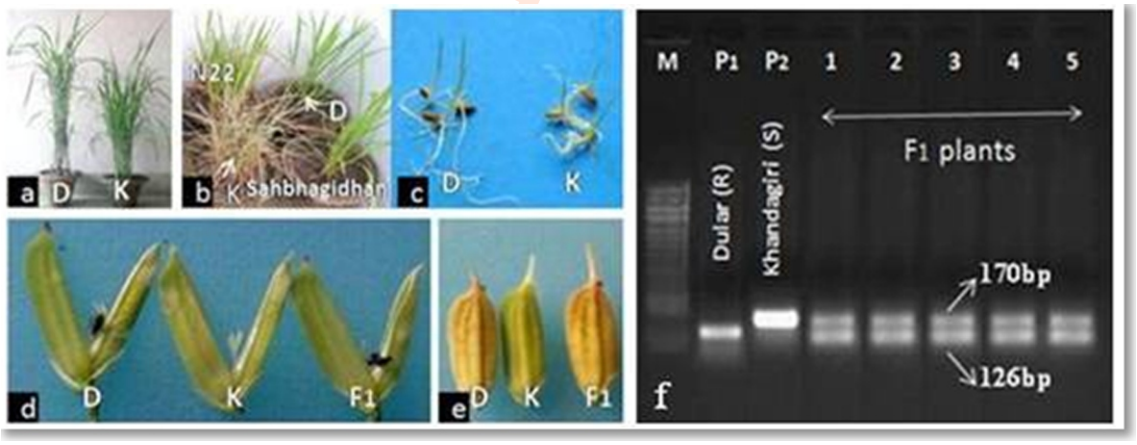

Fig. 1: Phenotyping of parents and F_1 in a cross "Khandagiri"(K) x "Dular" (D). N22 and Sahbhagidan are drought tolerant checks; Images a to e show parental polymorphism (a: tall vs semi-dwarf, b: drought tolerance vs drought sensitive, c: deep root vs short fibrous root system, d: black pigmented stigma vs greenish white stigma, e: presence vs absence of epiculous pigmentation in cv. Dular and cv. Khandagiri respectively). F_1 showed dominant traits present in cv. Dular. f: Molecular profiling of parents and F_1 plants using drought stress linked SSR marker RM 8085 (on chromosome 1). M: 100bp Mol. Wt. marker, P_1: Dular (drought resistant), P_2: Khandagiri (drought sensitive), Lane 1-5: F_1 plants.

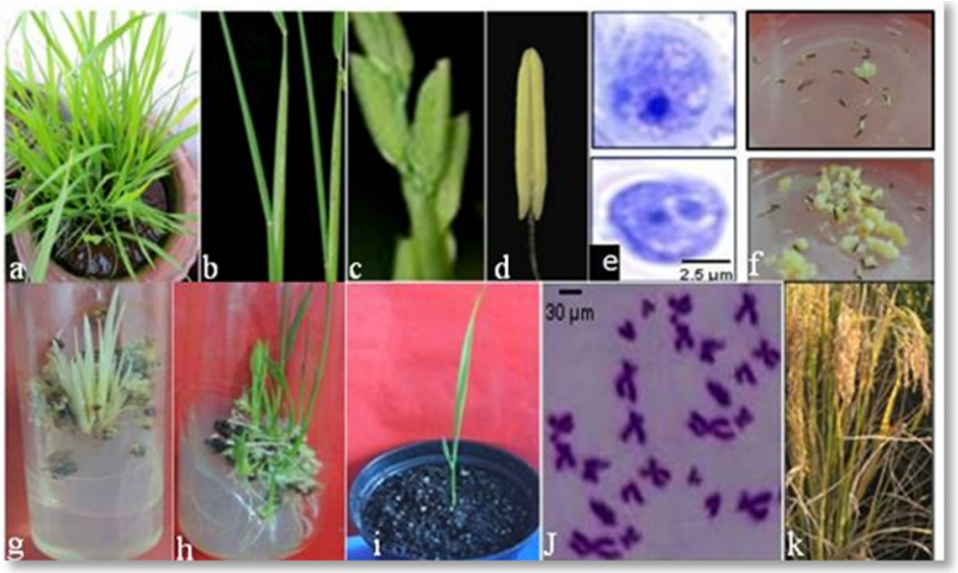

Fig. 2: Anther culture derived plantlet production in a rice cross "Khandagiri x Dular". a) Rice plant as source of explants, b) Boots, c) Spikelets, d) Anther, e) late uninucleate (upper) and early binucleate (lower) microspore, f) Callus induction in primary culture after 21 days (upper) and rapid growth of calli at 30 days (lower) in CIM + 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn, g) Albino plant regeneration in RM + 0.25 mg l^{-1} Kn + 0.75 mg l^{-1} BAP + 0.25 mg l^{-1} NAA, h) Green plant regeneration with rooting in RM + 2.0mg l^{-1} BAP + 0.5 mg l^{-1} NAA, i) Plant establishment in pot mixture, j) Prometaphase plate of root tips of a doubled haploid with 12 pairs of chromosomes, K) Field grown plants at maturity stage.

Selection of parents and raising F_1: Dular was characterised by tall plant type, deep root system, tolerant to moisture deficit (similar to N22 and Sahbhagidhan) and pigmented (black) stigma, while Khandagiri (drought sensitive) had semi-dwarf plant type with short fibrous root system and greenish white stigma similar to most of the rice varieties (Fig 1). In addition, Dular retained epiculous pigmentation in matured grains following dough stage, but it was absent in Khandagiri. Pigmented stigma (Lei *et al.,* 2006) and epiculous pigmentation (Akbar *et al.,* 1975) on grain in Dular are dominant traits. Hence, plants raised from crossed seeds which revealed both such dominant traits were confirmed to be genuine cross. Besides, the presence of both drought sensitive (170bp) and drought tolerance allele (126bp) in the F_1 plants following PCR with SSR marker RM 8085 (Fig. 1) further confirmed the success in hybridization.

Anther culture response for callus induction: Success of androgenesis depends on genotype, physiological status of donor plant, pollen development stage, media composition, culture incubation conditions and anther pre-treatments (Ruwani *et al.,* 2018; Tripathy *et al.,* 2019). N6, MS and SK1 solid media are in vogue used for anther culture and the former being reported to be more potent than two later media (Siddique, 2015). 2,4-D and NAA alone or with kinetin in the culture medium seem to be the major determinants for callusing from rice anthers (Herath *et al.,* 2008; Lal *et al.,* 2014; Mukherjee *et al.,* 2015). In the present investigation, pollen grains from middle spikelets of the panicles collected from pot grown mother plants were checked for proper stage of pollen development (late uninucleate to early binucleate) prior to anther culture (Fig. 2a-e). The anther culture response was assessed in both the parents (Khandagiri and Dular) and their F_1 hybrid using N6, SK3, MS and CIM medium (Table 1) supplemented with different concentration of 2,4-D, Kn and NAA. Media, genotypes, hormonal treatments as well as their inter se interactions were shown to be significant, except Genotype x Treatment (hormone recipe) interaction (Table 2). This means that both the parents and their F_1 revealed almost similar pattern of response to different hormonal recipes for anther culture, although there was significant difference among treatments and genotypes over different media. N6 was originally formulated for anther culture. In general, SK3 (a modification of N6) recorded higher callus induction frequency (12.93%) than N6 (6.53%) as well as MS (7.70%) tested over Khandagiri, Dular and their F_1 hybrid (Table 3). However, CIM (a formulated modification of MS) had revealed excellent callusing response (16.66%) with creamy nodular (embryonic) calli than all other media used due to higher content of nitrogen (in form of KNO_3) (Raina, 1997), iron and sucrose (6%) (Nistch, 1972) and inclusion of glutamine (500 mg l^{-1}), tryptophane (100 mg l^{-1}), cysteine (40 mg l^{-1}), proline (200 m l^{-1}) and casein hydrolysate (500 mg l^{-1}) (Tripathy *et al.,* 2019) in the CIM medium. The reason may be due to the fact that potassium nitrate, chelated iron (FE-EDTA) and sucrose are crucial for pollen embryo development (Nitsch, 1972; Raina, 1997). Besides, casein hydrolysate is a source of readily available calcium, several micronutrients, vitamins and amino acids (George *et al.,* 2008). Ali *et al.* (2021) formulated a modified N6 medium (A1) with reduced

Table 1: Composition of five minimal media used for anther culture in upland rice

Components	Concentration of basic components (mg l^{-1})				
	N6	SK3	MS	CIM	RM
NH_4NO_3	-	-	1,650.0	1,650.0	1650
KNO_3	2830.0	2830	1,900.0	2250.0	1900
$MgSO_4$, $7H_2O$	185.0	280	370.0	370.0	370
$MnSO_4$,$4H_2O$	4.4	4.4	22.3	22.3	22.3
$ZnSO_4$, $7H_2O$	1.5	1.5	8.6	8.6	8.6
$CuSO_4$, $5H_2O$	-	-	0.025	0.025	0.025
$(Nh4)_2SO_4$	463.0	315	-	-	232
$CaCl_2$,$2H_2O$	166.0	166	440.0	440	400
KI	0.8	0.8	0.83	0.83	0.83
$CoCl_2$, $6H_2O$	-	-	0.025	0.025	0.025
KH_2PO_2, $7H_2O$	400.0	640	170.0	170.0	170.0
H_3BO_3	1.6	1.6	6.2	6.2	6.2
Na_2MoO_4,$2H_2O$	-	-	0.25	0.25	0.25
$FeSO_4$,$7H_2O$	27.8	55.5	27.85	77.8	55.7
Na_2EDTA,$2H_2O$	37.3	74.5	37.25	104.3	74.5
Myo-inositol	-	-	100	100	100
Nicotinic acid	0.5	2.5	0.5	0.5	0.5
Pyridoxine HCl	0.5	0.5	0.5	0.5	0.5
Thiamine-HCl	1.0	0.5	0.1	0.5	0.25
Glycine	2.0	10	2.0	2.0	2.0
Glutamine	-	-	-	500	500
Tryptophan	-	-	-	100	100
Cysteine	-	-	-	40	40
Casein hydrolysate	-	-	-	500	500
Adenine sulphate	-	-	-	-	200
Proline	-	-	-	200	500
Agar	6,000	7000	8,000.0	8,000	8,000
Carbon source	60,000 Sucrose	60,000 Maltose	30,000.0 Sucrose	60,000 Sucrose	60,000 Sucrose
pH	5.7	5.7	5.8	5.8	5.8

concentration of 2, 4-D (1 mg l^{-1}), NAA (1.0 mg l^{-1}), Zn SO4 (1.5 mf l^{-1}) and addition of zeatin (0.10 mg l^{-1}), 100 mg myo-inositol, 3% sucrose and 3% maltose that revealed excellent callusing response in two rice hybrids (CXYR 24 and Y2). Similarly, Silva (2010) reported increased anther culture response in indica rice at higher concentration of nitrogen, phosphorus and potassium. Besides, supplementation of organic additives, e.g., yeast extracts, casein hydrolysate and coconut water to N6 media is reported to show enhanced androgenic callus induction in indica rice varieties (Roy and Mandal, 2005).

The success behind high frequency callusing response achieved by above researchers might be due to favourable genotype x medium interaction. In this study, 0.5 mg l^{-1} 2,4-D+2 mg l^{-1} Kn + 3.5 mg l^{-1} NAA, 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn and the auxin 2,4-D alone at 3.5 mg l^{-1} and 4.0 mg l^{-1} induced better callusing response across all the media and genotypes used (Table 3). Combination of 2,4-D, NAA and Kn to the anther culture media was also attempted earlier by Mukherjee *et al.* (2015). However, Niroula and Bimb (2009) reported higher callus induction frequency in N6 medium with 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn than N6 + 4 mg l^{-1} NAA + 0.5 mg l^{-1} Kn. Anther culture of F_1 plants were in fact targeted for double haploid production via callus induction. Among above highly responsive hormone recipes, CIM with 0.5 mg l^{-1} 2,4-D + 2 mg l^{-1} Kn + 3.5 mg l^{-1} NAA exhibited excellent callusing response (36.5%) in F_1, but induction of calli was delayed (after 45 days in primary culture) and became necrotic within few days even after subculture in the same media. In contrast, significantly higher callus induction (30.2% in F_1) with nodular calli amenable for plant regeneration was noticed as early as 21st day of primary culture in CIM supplemented with 1.5mg/l 2,4-D + 0.5mg l^{-1} Kn (Fig. 2f). Higher concentration of 2,4-D (4 mg l^{-1}) alone though induced statistically at par callusing response (32.5% in F_1) with 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn, but it may trigger chromosomal abnormalities and interfere in regeneration of plantlets in follow-up stages. Similar status of callusing response was noticed in both parents although Khandagiri excelled over Dular, while F_1 induced higher callusing ability than either of the parents (Table 3) across all media with

Table 2: Analysis of variance for callusing response in anther culture of parents (Khandagiri and Dular) and their F1 hybrid on four different media at varying hormonal recipes

Source	df	SS	MSS	F-value	SE(m)	CD5%	CV%
M	3	8426	2808.685	18506.70*	0.03	0.10	3.55
G	2	1141.173	570.587	3759.65*	0.03	0.08	
M x G	6	47.757	7.959	52.45*	0.06	0.17	
T	13	12445.7	957.362	6308.15*	0.06	0.18	
M x T	39	5254.369	134.727	887.73*	0.13	0.36	
G X T	26	-844.986	-32.499	-214.14 NS	0.11	0.31	
M x G xT	78	22.286	0.286	1.88*	0.22	0.63	
Error	336	50.993	0.152				
Total	503						

*Significant at P0.05; M: Media (N6, SK3, MS and CIM), G: Genotypes (Khandagiri, Dular and their F1), T: Treatments (14 hormonal combinations and concentrations are cited in Table 3)

maximum being revealed in CIM. Hence, CIM supplemented with 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} Kn was considered ideal for callus induction as also reported earlier (Tripathy *et al.,* 2019); and as such large-scale primary culture using huge numbers of anthers from F_1 plants was carried out in this media to produce sufficient number of callus clumps for follow-up study of plant regeneration response. This means that media inducing highest callusing frequency may not be considered always ideal so far as nature and age of calli is concerned for high throughput plant regeneration. Herath et al. (2007) recorded highest callus induction frequency (29.4%) in N6 medium for F_1 hybrid "Hu Lo Tao × BG 90-2" among a set of Japonica x indica hybrids. Dash et al. (2014) reported a callus induction frequency of as high as 37.83 % from anther culture of a cross "CRMS31B x CRMS24B". Besides, Thuan *et al.* (2001) reported better callus induction from anthers of F_1 plants derived from four crosses of aromatic and improved rice cultivars cultured in N6 and MS media with 2,4-D (0.5 mg l^{-1}) + NAA (1.0 mg l^{-1}) + BAP (0.5mg l^{-1}).

Anther culture response for plantlet regeneration: Albinism in regenerants is a serious problem in indica rice than japonica rice (Tripathy *et al.,* 2018; Lopez-Cristoffanini *et al.,* 2018). Besides, Indica x indica hybrids produce more of albino plantlets compared to indica x japonica hybrids (Herath *et al.,* 2010) possibly due to lower levels of the peroxidase enzyme in the former (Subhadra and Reddy, 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 (Tripathy *et al.,* 2019). Induction of sporophytic haploidy in rice anther culture is in vogue controlled by haploid (gametophytic) inhibitor gene 'hap' (which is activated by cold pre-treatment. This reverses the gametophytic development of the pollen grain to sporophytic status. Further, Kiruchi *et al.* (2003) reported that androgenesis in rice may be due to activation of a new class of Miniature Inverted-repeat Transposable Elements (MITE: mping elements) in anther derived calli. A rice genotype IR58025B with eui (25eB) gene is reported to be highly responsive for both callus induction and green plantlet regeneration among 13 genotypes (Kaushal *et al.,* 2014). The calli developed from anthers of F_1 plants in CIM with 1.5mg/l 2,4-D + 0.5 mg l^{-1} Kn were transferred to the regeneration medium (a modification of MS) with different combination of Kn, BAP and NAA for plantlet regeneration. In the present investigation, Kn or BAP alone or even their combinations did not elicit any plant regeneration response (Table 4). However, inclusion of NAA with BAP or combination of BAP and Kn revealed plantlet regeneration. Among different combination of hormone recipes, 0.25 mg l^{-1} Kn + 0.75 mg l^{-1} BAP + 0.25 mg l^{-1} NAA induced highest frequency of plantlet regeneration (18.2%) followed by 0.75 mg l^{-1} Kn + 0.75 mg l^{-1} BAP + 0.5 mg l^{-1} NAA (15%) in as early as 28-30 days of inoculation of calli in regeneration medium, but almost all such plantlets were albinos (Fig. 2g). Besides, most of the treatments, excepting a few, also induced higher frequency of albino plantlets with or without rooting. Gueye and Ndir (2010) also reported recovery of 79 albino plants out of total 93 regenerants in anther culture. This seems to be a major setback in the prospect of anther culture in genetic improvement of rice (Tripathy *et al.,* 2018; Roy and Mandal, 2005). However, it is interesting to note that the highest frequency of green plantlet regeneration (12.8%) with rooting was achieved within four weeks in RM + 2 mg l^{-1} BAP + 0.5 mg l^{-1} NAA without any albino plantlet formation (Fig 2h). The above media also resulted highest number (6 nos.) of green plantlets per responsive callus. In fact, the process of dedifferentiation and redifferentiation involves activation and/or suppression of several metabolic pathways (Biswas and Mandal, 2007). Such an instance may be achieved at a suitable hormonal combination and concentration.

This could be the reason for achieving high frequency of plant regeneration in the above hormone recipe. Besides, 2 mg l^{-1} Kn+0.5 mg l^{-1} NAA in RM medium also induced 3-4 green plantlets/responsive callus. Rout and Sharma (2001) reported consistently high frequency of plantlet regeneration from anther derived calli of a wide range of genotypes in MS medium with 10% coconut milk (CM) and addition of Kn (0.5 mg/1), BAP (2 mg/1) and NAA (1 mg/1) at ratio of 1:4:2. Xa and Lang (2011) reported 6.17% to 14% regeneration from four crosses in MS medium with combination 1 mg l^{-1} BA + 2 mg l^{-1} Kn + 3% sucrose.

Table 3: Callusing response in anther culture of parents (Khandagiri and Dular) and their F_1 hybrid on four different media at varying hormonal concentrations (mg l^{-1})

Hormone recipe (mg l^{-1})			Callus induction frequency (CIF %)												
2,4-D	Kn	NAA	N6			SK 3			MS			CIM			Mean
			P_1	P_2	F_1	P_1	P_2	F_1	P_1	P_2	F_1	P_1	P_2	F_1	
0.0	0.0	2.5	2.0	1.8	2.2	10.6	8.8	12.8	1.6	0.8	1.2	5.2^{gh*}	5.0^{h}	5.5_{g}	4.8
0.0	0.5	2.5	4.2	4.0	5.5	12.2	9.2	14.6	3.0	2.5	3.0	5.0^{gh}	4.8^{h}	6.6_{g}	6.2
0.5	1.0	2.5	5.5	4.5	6.9	10.9	10.0	14.8	10.0	1.3	12.4	18.0^{d}	16.8^{e}	20.2_{d}	10.9
0.5	2.0	3.5	8.5	6.8	12.8	14.8	15.0	20.2	14.2	12.0	18.4	31.6^{a}	27.8^{a}	36.5_{a}	18.2
1.5	0.0	0.0	6.4	4.8	8.8	8.9	7.8	12.4	6.9	4.5	10.2	18.0^{d}	15.0^{ef}	20.0_{d}	10.3
1.5	0.5	0.0	10.2	6.9	14.3	16.8	12.8	22.5	13.8	10.8	14.6	28.0^{b}	24.4^{bc}	30.2_{b}	17.1
1.5	0.5	1.5	4.0	3.2	6.0	6.8	5.6	10.2	6.9	7.0	10.4	14.8^{e}	14.0^{f}	15.8_{e}	8.7
2.5	0.0	0.0	6.2	6.0	8.9	10.9	10.6	14.3	10.6	10.0	12.0	23.0^{c}	22.8^{c}	24.0_{c}	13.3
2.5	1.0	0.0	8.8	6.6	10.5	15.6	15.2	16.2	9.0	8.8	8.8	14.4^{e}	15.0^{ef}	18.5_{d}	12.3
2.5	1.0	2.5	5.0	3.5	4.8	6.6	4.4	6.9	6.0	4.8	5.9	10.0^{f}	8.8^{g}	10.0_{f}	6.4
3.5	0.0	0.0	10.4	8.2	14.3	17.8	14.4	18.3	13.9	10.0	14.3	24.0^{c}	20.6^{d}	25.8_{c}	16.0
4.0	0.0	0.0	12.0	10.2	15.2	16.2	13.8	20.5	12.8	9.8	14.8	30.4^{a}	25.8^{ab}	32.5_{b}	17.8
0.0	0.0	3.5	1.5	1.4	2.5	13.3	10.6	15.6	1.4	0.9	1.6	4.0^{gh}	4.0^{h}	5.5_{g}	5.2
0.0	0.0	4.5	3.2	2.2	3.2	15.0	12.2	18.2	0.8	0.8	1.0	6.2^{g}	4.5^{h}	6.8_{g}	6.2
Treatment Mean			**6.3**	**5.0**	**8.3**	**12.6**	**10.7**	**15.5**	**7.9**	**6.0**	**9.2**	**16.6**	**15.0**	**18.4**	**10.96**
Media mean				**6.53**			**12.93**			**7.70**			**16.66**		**10.96**

N.B: P_1-Khandagiri, P_2- Dular, F_1(Khandagiri x Dular), * Means followed by same letter within columns were considered not significantly different at $P \leq 0.05$.

Table 4: Plantlet regeneration response from anther culture of a F1 cross 'Khandagiri x Dular' in regeneration media (RM)

Hormone recipe (mg l^{-1})			Days first plantlets observed in RM	Plantlet regeneration response (%)*			No. of plantlets/ responsive callus		Rooting status of plantlets
Kn	BAP	NAA		Green	Albino	Total	Green	Albino	
1.0	2.0	1.0	38	0.0	3.8	3.8de	0.0	1.57	No rooting
0.5	1.0	0.5	33	0.2	6.2	6.4cd	1.01	2.3	-do-
0.25	0.5	0.25	31	0.0	2.6	2.6e	0.0	3.1	Rooting
0.25	0.75	0.25	28	0.4	17.8	18.2a	1.85	12.1	-do-
0.5	2.0	1.0	38	1.3	2.5	3.8de	1.05	3.0	No rooting
1.0	2.0	0.5	32	0.5	6.3	6.8c	1.08	2.5	Less Rooting
2.0	0.0	0.5	30	2.8	5.2	8.0c	3.58	6.0	-do-
0.0	2.0	0.5	30	12.8	0.0	12.8b	6.1	0.0	Normal rooting
0.0	2.0	0.0	No response	0.0	0.0	0.0e	0.0	0.0	-
2.0	0.0	0.0	No response	0.0	0.0	0.0e	0.0	0.0	-
0.75	0.75	0.5	30	1.5	13.5	15.0b	1.23	10.1	Rooting
1.0	1.0	0.5	35	0.6	5.2	5.8cd	0.0	1.5	-do-
1.5	0.5	0.0	No response	0.0	0.0	0.0e	0.0	0.0	-
0.5	1.5	0.0	No response	0.0	0.0	0.0e	0.0	0.0	-

*% of anthers responded to plantlet regeneration in RM medium. * Means followed by the same letter within columns were considered not significantly different at $P \leq 0.05$

Recovery of double haploid lines: In rice, seventy percent of the anther derived regenerated plants usually show haploid status, while the remaining accounts in vitro euploids (including spontaneous diploids) and aneuploid. However, Germana (2011) reported spontaneous chromosome doubling (endoreduplication) of 50-60% of haploids in rice anther culture. Thus, in rice, chromosome doubling by "colchicine" (a mitotic inhibitor) may be omitted unless desired for increased doubled haploid production. Further, colchicine treatment is usually avoided in rice to reduce chances of mixoploids and polyploids resulting chimeric plants and low seed set at whole plant level (Tripathy, 2018). In this study, plantlets were grown in pot mixture (Fig 2i) and only 335 plantlets survived after acclimatization in the green house. However, 129 plants (38.5%) out of 335 anther culture derived plantlets from the rice hybrid 'Khandagiri x Dular' revealed spontaneous double haploid (DH) status, each with 12 pairs of chromosomes (Fig. 2j). These anther culture derived plants maintained normal growth without any abnormality and set seeds upon flowering (Fig. 2k).

The remaining plantlets either did not sustain plant establishment in field due to weak growth or abnormal growth without flowering (sessile) and many were found to be sterile. Progenies within each of the 129 DH lines resulted from above doubled haploid plants exhibited uniformity in morpho-agronomic traits. Sellamuthu *et al.* (2011) recovered a doubled haploid population targeted for selection under drought. Xa and Lang (2011) recovered 133 DH lines out of which 22 outstanding DH lines were selected for yield and grain quality. Similarly, Allah *et al.* (2014) successfully recovered forty anther culture derived lines from five crosses *viz.,* Giza 177 x IET 1444, Giza 177 x Yun Len 4, Sakha 101 x IRAT 112, Sakha 103 x IET 1444 and Sakha 103 x Suweon 349. Purwoko *et al.* (2010) recovered 92 doubled haploid lines from 13 crosses out of which 24 lines had high seed yield with tolerance to biotic and abiotic stresses. Anther culture is an amazing biotechnological tool for production of fixed breeding lines (doubled haploid lines) from intervarietal and interspecific crosses. In vogue, indica sub-group is recalcitrant to androgenesis and there is narrow genetic variation for anther culture response in Asian rice. Very low callusing response and follow-up recovery of higher frequency of albino plants limit the progress of double haploid breeding in indica rice.

Genetic basis of anther culture response is still not clear. A number of research initiatives have been piled up for optimization of anther culture response in this crop, but none of the recent findings seem to be genotype-independent. However, a highly reproducible green plantlet regeneration system explored in this study can be amenable to recover homozygous plants (doubled haploids) with rare gene combinations bypassing the normal selfing in F_1 plants, and as such can accelerate the breeding process for isolation of high yielding plant types with biotic and abiotic stress tolerance in upland rice.

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