Marker aided selection of double haploids for drought tolerance in rice

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CERTIFICATE I

This is to certify that the thesis entitled **"Marker aided selection of double haploids for drought tolerance in rice"** submitted in partial fulfillment of the requirements for the award of the degree of **Master of Science in Agriculture (Agricultural Biotechnology)** to the Orissa University of Agriculture and Technology is an faithful record of *bona fide* and original research work carried out by **Miss Manasmita Maharana (Adm. No. 08ABT/15)** under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

It is further certified that the assistance and help obtained by her from various sources during the course of investigation has been duly acknowledged.

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CERTIFICATE II

This is to certify that the thesis entitled **"Marker aided selection of double haploids for drought tolerance in rice"** submitted by **Miss Manasmita Maharana (Adm. No. 08ABT/15)** to Orissa University of Agriculture and Technology, Bhubaneswar in partial fulfillment of the requirements for the degree of **Master of Science in Agriculture (Agricultural Biotechnology)** has been approved by the students' Advisory Committee and the external examiner.

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Bhubaneswar (Manasmita Maharana)
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ABSTRACT

Anther culture is *in vogue* used for production of purely homozygous lines. The aim of this investigation was to develop drought resistant double haploids from a cross as an attempt to shortcut breeding cycles for development of rice varieties. Embryogenic callus was successfully induced from anthers of F_1 plants of. a cross Khandagiri (an improved high yielding upland rice variety) x Dular (a drought tolerant local land race). Three standard basal media i.e., N6, SK3 and MS and a formulated callus induction medium (CIM: a modification of MS) with different hormone recipes (2,4-D, Kn and NAA) were tested for callus induction. CIM medium supplemented with 1.5mg/l 2,4-D + 0.5mg/l Kn induced highest percentage (29.5%) of nodular friable embryogenic callus ideal for plantlet regeneration. A regeneration medium (RM) with Kn, BAP and NAA at different concentration was also prepared as a modification of MS medium to suit plantlet regeneration from anther derived calli. Embryogenic calli derived from above highly responsive callus induction media (MS + 1.5mg/l 2,4-D + 0.5mg/l Kn) revealed highest green plantlet regeneration response(12.8%) with an average of six plantlets per responsive callus in RM media with 2mg/l BAP + 0.5mg/l NAA. Well developed green shoots were transferred to MS medium without phytohormones for root initiation. These plantlets were initially transferred to half-strength MS basal liquid medium for one week (for primary hardening) before transfer to pots filled with mixture of peat moss: perlite 2:1 (v/v). Altogether 55 putative DHs out of total 123 anther derived plantlets came to normal flowering and produced filled grains. These DHs were tested for their status of drought tolerance under artificial moisture stress using PEG_{6000} at seedling stage and in pot culture at vegetative and panicle initiation stage. Considering different phenotyping criteria for drought tolerance e.g., extent of root growth, leaf drying, leaf rolling and drought recovery (after re-watering); DHL 1, DHL 4, DHL 8, DHL 24, DHL 30, DHL 41, DHL 43, DHL 48 and DHL 53 were considered drought resistant. Besides, molecular profiling of genomic DNA was carried out using a gene specific SSR primer (RM 8085 in Chromosome 1) for both the parents to assess parental polymorphism and to assist selection of drought resistant plants in the present set of double haploid population. Dular (drought resistant donor) revealed the resistant allele of amplicon size 126bp while Khandagiri had shown a 170bp amplified product. Molecular profiling of the above DHLs revealed the 126bp allele which confirms their drought tolerance status. Among these double haploid lines, DHL 53, DHL 41, DHL 24 and DHL 4 recorded significantly higher seed yield than Sahbhagidhan and Mandakini (standard checks).This investigation has enriched the knowledge of both embryogenic callus induction and plantlet regeneration in upland rice and has lead to the development of pure, double-haploid lines for use in rice breeding programme in OUAT.

ABBREVIATIONS

%	:	Percentage
@	:	At the rate
°C	:	Degree celsius
µl	:	Micro liter
µM	:	Micro molar
µmol	:	Micro mole
β-ME	:	β- Mercapto ethanol
2,4-D	:	2,4-dichloro-phenoxyacetic acid
ABA	:	Abscisic acid
AFLP	:	Amplified fragment length polymorphism
$AgNO_3$	:	Silver nitrate
ATP	:	Adenosine tri-phosphate
BAP	:	6 benzyl aminopurine
BC_2	:	Second backcross
BILs	:	Back cross inbred lines
bp	:	Base pair
BSA	:	Bulk Segregant Analysis
CIF	:	Callus induction frequency
CIM	:	Callus Induction Medium
cM	;	Centimorgan
cm	;	Centimeter
CSSLs	:	Chromosomal segment substitution lines
CTAB	:	N-Cetyl-N, N, N-trimethyl ammonium bromide
DAS	:	Days after sowing
DF	:	Days to 50% flowering
DH	:	Double haploid
DHL	:	Double haploid line
DM	:	Days to maturity
DMSO	:	Dimethylsuphoxide
DNA	:	Deoxy ribonucleic acid
dNTPs	:	Deoxynucleotide
DRS	:	Drought recovery score

EBT	:	No. of effective bearing tillers/m^2
ECR	:	Embryogenic callusing response
ELS	:	Embryo-like structure
ERR	:	Embryogenic regeneration response
F%	:	Fertility percentage
F_1	:	Firstfilial generation
F_2	:	Second filial generation
Fe-EDTA	:	Chillated ethylene diamine tetra acetate
Fig	:	Figure
G/P	:	Number of grains per panicle
G	:	Gram
GW	:	1000-grain weight
Ha	:	Hectare
HCl	:	Hydrochloric acid
H	:	Hour
HSPs	:	Heat shock proteins
IAA	:	Indole acetic acid
IRRI	:	International rice research institute
ISSR	:	Inter Simple equence repeat
Kb	:	Kilo base pair
KCl	:	Pottasium chloride
Kn	:	Kinetin
L	:	Litre
LRS	:	Leaf rolling score
M	:	Molar
MAGIC	:	Multi-parent advanced generation intercrosses
MAS	:	Marker-assisted selection
$MgCl_2$	:	Magnesium chloride
Mg	:	Miligram
Min	:	Minute
MITE	:	Miniature Inverted-repeat Transposable Elements
M	:	Meter
mM	:	Milli mole
MPRILs	:	Multiparent recombinant inbred lines

MS	:	Murashige and Skoog medium
NAA	:	Napthalene acetic acid
NaCl	:	Sodium chloride
NAM	:	Nested association mapping
NaOH	:	Sodium hydroxide
NH_4^+	:	Ammonium
NILs	:	Near isogenic lines
Nm	:	Nanomole
PCR	:	Polymorphic chain reaction
PEG	:	Poly ethylene glycol
PHT	:	Plant height
PL	:	Panicle length
ppm	:	Parts per million
PVP	:	Polyvinylpyrrolidone
QTL	:	Quantitative trait loci
RAPD	:	Random Amplification of Polymorphic DNA
RILs	:	Recombinant inbred lines
RM	:	Regeneration medium
ROS	:	Reactive oxygen species
rpm	:	Revolution per minute
SD	:	Segregation distortion
s	:	Second
SSRS	:	Simple sequence repeat
SY/P	:	Seed yield per ha
TE	:	Tris $_{10}$ EDTA$_1$
t	:	tonne
UV	:	Ultraviolet
β-ME	:	β-mercapto ethanol

INTRODUCTION

Rice (*Oryza sativa* L, 2n=24, Family Poaceae) is one of the most important food crop of the developing world. It feeds over half of the global population (Sasaki, 2005). About 90% of the world's rice is produced and consumed in Asia. It provides up to two thirds of calories for more than two billion people in Asia. Nearly 100 million people now depend on upland rice as their daily staple food. Almost two-thirds of the upland rice area is in Asia. Bangladesh, Cambodia, China, India, Indonesia, Myanmar, Thailand, and Vietnam are important producers. Many upland farmers' plant local rice that do not respond well to improved management practices - but these are well adapted to their environments and produce grains that meet local needs.

Genetic improvement of rice in India and many other rice growing countries has already achieved yield platuae for medium land irrigated rice ecosystems, but areas pertaining to rainfed rice ecosystems particularly high land situations are constrained with low productivity owing to erratic rainfall distribution. The existing genotypes particularly the short duration rice varieties so far developed are rarely adaptable to pre and post-monsoon drought situation. It has been contemplated that climatic change will compel rice breeders to reorient the breeding strategies and develop high yielding short duration rice varieties to combat the recurrent occurrence of drought in coming years.

Drought is the predominant cause of yield reduction in rain fed rice production systems, but until recently, little systematic effort has been made to breed drought-tolerant cultivars. The complex nature of drought tolerance, genotype × environment interaction, and the difficulty of effective drought tolerance screening complicate the development of drought tolerant varieties. Several varieties that have been widely grown in rain fed environments in India for many years are indeed lack of drought tolerance leading to low productivity. The cultivation of upland rice is a non-flooded alternative to lowland rice culture that can reduce the demand for irrigation water by 50–70%. Rice varieties adapted specifically to upland conditions are typically characterized by a deep root system, tall stature, thicker stem and fewer tillers. These characteristics are believed to confer adaptability to the upland conditions. It is reported that the long and thick root system of upland rice contributes greatly to its

drought resistance. Additional studies have shown that the ratio of root weight to shoot weight, and root penetration ability is also correlated with drought resistance.

Water deficit reduces crop yield regardless of the growth stage and varieties owing to energy (ATP) drain to combat drought which could otherwise be utilized to increase productivity. Major effect of drought in plant is reduction in photosynthesis, which is due to decrease in leaf expansion, impaired photosynthetic machinery, leaf senescence and finally reduction in assimilate production and partitioning. Water deficit in root zone causes an increase in rate of root respiration which leads to an imbalance in the utilization of carbon resources, decrease in production of adenosine triple phosphate (ATP) and an increase in production of reactive oxygen species (ROS). Tall stature cultivars produced more leaf area, aerial biomass; so they transpired more water. While, the dwarf/intermediate tall cultivars particularly with deep root system could initiate osmotic adjustment earlier than tall stature cultivars.

Drought stress (due to soil drying) caused plants to produce abscisic acid in roots. ABA produced in dehydrated roots, transported via the transpiration stream to the leaves through xylem and regulates stomatal opening and leaf growth in the shoots. Concentration of ABA in the guard cell apoplast increased as a stress signal. It activates a set of protein molecules called receptors. These receptors then activate a series of changes to help the plant survive under drought stress. The natural reaction of the receptors is to close the guard cells on and inside the leaves. That closure decreases water loss and stops the vegetative parts from growing to save water during a drought, but root growth appears to be promoted which increased the water supply. Now a days, the genes for ABA receptors can be engineered to tune increased closure of guard cells. Hundred versions of the ABA receptor protein genes are being tested until ones found work better. Besides, status of drought tolerance depends upon changes of Rubisco and some stress inducible proteins (chaperons, HSPs, DHNs and proteases) and their complex interrelationships under drought and control conditions. Dehydrin protects cytosolic structures from the deleterious effects of cellular dehydration.

Creation of genetic variability is essential in any crop improvement programme. Breeders often resorted to hybridization or mutation breeding and even genetic transformation for genetic improvement of specific trait(s). Several conventional breeding methods are now available which take 8-10 years to develop a fixed (homozygous) breeding line. However, anther culture technique encompasses

creation of usable genetic variability and increases the selection efficiency to obtain quick homozygosity from even F_1. It provides fertile double haploids (DHs) with fixed derived character combination that might otherwise disappear in the course of an extended series of segregating generations in conventional breeding methods. Besides, the characters those expressing predominantly additive genetic effects, their values in DH lines could reach the heterotic level of the hybrid (Ba Bong and Swaminathan, 1995) which further pinpoint the prospect of anther culture technique in upland rice. The DH lines derived from anther culture of hybrids of genetically diverse parents are amenable for molecular mapping of valuable genes (simply inherited traits) and QTLs for agro-economic and quality traits in rice. Identification of useful markers linked to QTLs for drought resistant trait(s) would help to develop drought tolerant rice varieties using marker assisted selection.

Therefore, the proposed work entails to develop drought tolerant rice varieties using marker aided selection of double haploids following crossing of a drought tolerant donor (Dular-a tall indica local land race) with a popular high yielding mega variety of rice (Khandagiri) under upland condition. Brief objectives are outlined as follows.

1. Development of double haploids using standardized protocol of anther culture
2. Validation and identification of most suitable polymorphic marker associated with
   drought tolerance.
3. Selection of double haploids for drought tolerance under artificial drought stress and using molecular markers
4. Phenotyping of selected drought tolerant DH lines for physiological and morpho-economic traits under drought stress.
5. Identification of high yielding drought tolerant DH line

REVIEW OF LITERATURE

Rice being highly self pollinated, development and selection of pure breeding lines with manifested superior phenotype is the ultimate objective for desired genetic improvement. It normally needs 6-9 cycles of selfing followed by 3-5 years of field evaluation. In contrast, anther culture seems to be a suitable technique that significantly reduces the breeding period due to early fixation of homozygosity (Otani *et al*., 2005). Genetic recombination occurs during micro-gametogenesis producing genetically unique male gametophyte. Anther culture induces haploid callus formation, which converted to immortal true breeding double haploid (DH) embryos due to spontaneous genome doubling. Consequently, each DH line obtained in this way would bypass the inbreeding process (Brar *et al*., 2006, Germana *et al*., 2011) and produce a new true breeding line with unique gene combination. This allows better discrimination between genotypes (Maria *et al*., 2006) resulting increased selection response. A sizeable proportion (70%) of plantlets in culture is *in vogue* haploids which either do not survive till maturity or become sterile due to abnormal meiotic behaviour during gametogenesis. However, these may be valuable to detect desirable recessive traits introduced through mutation (Chen *et al*., 2001) or hybridization (He *et al*., 2006) and such plantlets may be converted to DHs by colchicines treatment. However, compared to other crops, spontaneous production of DHs at high frequency in *in vitro* anther culture is a regular phenomenon in rice.

Anther culture offers greater chance of recovery of otherwise desirable recessive genes compared to conventional breeding. Double haploid breeding involve only one cycle of meiotic recombination. However, many agronomic traits are polygenically controlled. One cycle of recombination is usually insufficient for the improvement of such quantitative traits since crossing over between polygenes will not release all potential variations available in the cross. To overcome these disadvantages, the Chinese developed a method combining anther culture with sexual hybridization among anther derived plants. The anthers of the hybrid (F_1) progeny are excellent breeding material for raising pollen-derived homozygous plants (double haploids) in which complementary parental characteristics are combined in one generation.

Varieties developed through anther culture can yield as high as 10.3t/ha under moderate fertility. It can be utilized for developing direct one-step homozygous transgenic plants (Chen *et al.*, 2006), increased selection efficiency, genetic manipulation, widening genetic variability through the production of gametoclonal variants and for production of double haploid populations and allowing early expression of recessive genes. Consumer's life style and demand for a suitable variety changes with time and therefore, anther culture can be a suitable alternative for genetic improvement in rice. A brief review on the above works follows.

2.1 Anther/pollen culture in rice

The accidendal discovery of androgenic haploidy in *Datura* by Guha and Maheswari (Guha *et al*., 1964) led several researchers to attempt such novel technique in a wide range of crop plants. Haploid plant production was first reported in rice through anther culture by Niizeki and Oono (Niizeki and Oono, 1968). Anther culture is a two step process. First step is the initial development of callus that leads to second step i.e regeneration of green plants from callus. Limited morphogenetic potential of anther derived calli and a higher percent of regenerated albino plants (Roy *et al*., 2005, Talebi *et al*., 2007) seem to be major constraints for double haploid breeding. Gueye and Ndir (Gueye and Ndir, 2010) reported recovery of a total of 93 regenerants out of which 79 were albinos. Many researchers have reported genotypic specificity within indica subspecies by using improved media (Talebi *et al*., 2007, Ratheika *et al*., 2007). Green plant regeneration is reported to be enhanced by six days of cold pretreatment to the panicles (Sen *et al*., 2011). Nirouli and Bimb (Nirouli and Bimb, 2009) reported higher callus induction frequency in N6 medium with 2.5mg/l 2,4-D(2,4-dichloro-phenoxyacetic acid) + 0.5mg/l Kinetin(Kn) than N6 + 4mg/l NAA(Napthalene acetic acid) + 0.5mg/l Kn; but reverse was the case for green plant regeneration. Xa and Lang (Xa *et al*., 2011) reported 5.13% to 9.27% callus induction and 6.17% to 14% regeneration from four crosses in MS medium with combination 1mg/L BA(6 benzyl aminopurine) + 2mg/L Kinetin + 3% sucrose. High frequency of callus induction was also obtained from incorporation of 2,4-D, NAA and kinetin to the anther culture media (Herath *et al*., 2008, Lal *et al*., 2014, Mukherjee *et al*., 2015). Profuse microtillering of androgenetic plantlets was found in elite indica rice variety IR 72 which can be further depressed by higher concentration of BAP (Roy *et al*., 2011). The F_1 hybrids are more responsive to anther culture than their parents. Herath

et al. (2007) recorded highest callus induction frequency (29.4%) in N6 medium for F_1 hybrid Hu Lo Tao × BG 90-2 with green plant regeneration frequency (41.0%) in MS medium from calli induced on N6 medium. However, positive relationship for both was noticed by Shahnewaz *et al*. (2004). Thuan *et al*.(2001) reported callus induction from anthers of F_1 plants derived from four crosses of aromatic and improved rice cultivars cultured in N6 and MS media supplemented with 2,4-D (0.5mg/L)+ NAA (1.0mg/L)+ BAP (0.5mg/L) showed better callus induction. Dash *et al*. (2014) reported a callus induction frequency of as high as 37.83 % from anther culture of a cross CRMS31B x CRMS24B when incubated at 26 ± 1^{o}C for 24hr.

2.2 Factors affecting anther culture

Success of anther culture depends upon exogenous and endogenous factors such as maturity of the donor plant (Afza *et al*., 2000), genotype (Ramakrishanan *et al*., 2005, He *et al*., 2006), microspore developmental stages (Afza *et al*., 2000), panicle pretreatment (Trejo-Tapia *et al*., 2002) temperature and duration of pretreatment (Kiviharju *et al*., 1998), culture media (Asaduzzaman *et al*., 2003), growth conditions (Raina *et al*., 1998).

2.2.1 ***Genotype***

Response to anther culture varies differently within species, subspecies, or varieties. *O. glaberrima* is more potent for callusing and regeneration than *O. sativa* (Gueye *et al*., 2010). Japonica types are in vogue more responsive to microspore embryogenesis than indica types in rice. Indica cultivars show poor callus growth, poor regeneration ability and low percentage of albino plants (Raina, 1997). Only 5 out of 18 indica cultivars showed pollen callusing and 4 calli differentiate into plants (Guha-Mukherjee, 1973) whereas, only one out of 35 indica cultivars exhibited pollen callusing (Lentini *et al*., 1995). Tran and Vuong (2002) also recorded low response for callus induction (3.53%) and plantlet regeneration (1.12%) frequency in indica rice. However, combining high yielding indica rice with high anther culture responding japonica genotype may improve anther culture response (Herath *et al*., 2011). Calli from a F_1 hybrid (Bg 90-2: indica rice ×Hu Lo Tao: japonica rice) showed higher frequency of green plantlet regeneration than their parents. High callus induction frequencies (30-34%) and low regeneration response in F1s was obtained by Mishra *et al*. (2011) and Talebi *et al*. (2007). However, positive relationship for both was noticed by Shahnewaz *et al*. (2004) and Javed *et al*. (2007).

2.2.2 *Physiological status of donor plant*

Anthers collected at the beginning of the flowering period respond better and it declines with age of plants (De Laat *et al*., 1987). Field grown plants show superiority over to those grown in the glasshouse or pots (Debata *et al*., 1989). Plants with excessive vegetative growth are not desirable and therefore, second application and/or foliar spray of nitrogen is avoided. Besides, a favourable day (34^oC) and night (25^oC) temperature at booting stage seems to be a determining factor for androgenic embryogenesis (Prem *et al.*, 2004). Anthers from the primary tillers are *in vogue* more responsive than secondary tillers (Asif, 2013). Besides, physiological status of anthers from middle portion of the panicles in boot stage seems to be favourable for callusing and regeneration (Jacquard *et al.*, 2006).

2.2.3 *Developmental stage of pollen*

The developmental stage of pollen can enhance anther culture efficiency. In rice, the most suitable stage is the early to mid- uninucleate pollen stage (Datta *et al*.,1998), while the older pollens at tetrad stage and after the first pollen mitosis do not respond to culture due to starch deposition leading to differentiation into male gametophyte (Cha-um *et al*., 2009; Silva, 2010). Such above appropriate condition correspond to the 3-4cm distance between the collar of the flag leaf and ligule of the penultimate leaf (Bishnoi *et al.*, 2000). Therefore, it is important to optimize the anther culture response in rice by assessing optimum developmental stage of pollen grain in the anther by acetocarmine staining.

2.2.4 *Pre-treatment*

Anthers exposed to various kinds of stresses (cold, heat, osmotic stress, sugar starvation, gamma irradiation and chemical treatment) prior to *in vitro* culture are reported to induce androgenesis (Shariatpanahi *et al*., 2006) and inhibit callus induction from somatic origin (anther wall and tapetum). However, the type and duration of pre-treatments vary with the species and variety of rice (Datta, 2001).

Cold pre-treatment: A low temperature stress has been reported to induce and ensure continuance of sporophytic mode of development of microspores (Matsushima *et al.*, 1988) instead of follow-up gamete formation. It triggers embryogenesis from microspores (Chen *et al*., 1991) and increases the frequency of spontaneous development of DH plants (Amssa *et al*., 1980). Pre-treatment of anthers at 10^oC for 11-12days (Gupta *et al*., 1987) results better callusing response but led to albino

plantlet regeneration while, pretreatment at 12^0C for 5days gives best regeneration response(Kaushal *et al*., 2014a; ,Sen *et al*., 2011). Besides, cold pre-treatment eliminates weak or non-viable microspores in culture (Lenka *et al*., 1994), delays anther wall senescence and increases symmetric division of pollen grains and release of necessary substances (cold shock proteins and amino acids) for androgenesis (Kiviharju *et al*., 1998).

Heat shock treatment: Heat temperature pre-treatment synchronizes physiological states of microspores and stimulates embryoid formation by disrupting the cytoskeleton at initial stage of microspore development (Ferrie *et al*., 1995).

Osmotic Stress: Osmotic stress e.g., mannitol can offer as a substitute or replace the pre-cold treatment as it enable physiological isolation microspores (Wetherell *et al*., 1984) leading to embryoid formation. It improves the sugar uptake causing increased glucose level in the anther tissue. Treatment of anthers in 0.4 M mannitol was useful to enhance androgenesis in anther culture of indica cultivar IR 43 from 3 to 33%. However, cold treatment combined with mannitol induced osmotic stress had no added advantage (Pande *et al*., 1997) or even may be detrimental.

Sugar starvation: Sugar starvation contributes to the promotion of high frequency embryogenesis and plantlet regeneration from microspores isolated from anthers of indica and japonica rice and also in isolated microspores (Raina *et al*., 1998). 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 (Ogawa *et al*., 1995). However, cold treatment was superior to sugar starvation. Touraev *et al*. (1996) reported better anther culture response by starvation of anthers in sugar-free medium in wheat.

Gamma radiation: Low irradiation pre-treatment is known to promote embryogenesis in anther culture. Chen *et al*. (2001) reported significant stimulatory effect of 20kR gamma ray treatment on regeneration of green plantlets. Similarly, Mkuya *et al*. (2005) observed prolific doubled haploid production and green plant regeneration following gamma-irradiation of indica rice line TM7-5.

Chemical treatment: Ethrel and ethephon (ethylene releasers) has pronounced effect in haploid production in various plant species. Plants are sprayed with 4000ppm ethrel just before meiosis in PMCs which results in multinucleated (4-6) pollen with fewer starch grains. Parthenogenic embryoids may be induced due to additional mitosis from such pollen grains in culture (Chawala, 2010). Wang *et al*. (1979) reported enhancement in anther culture response with 4000ppm ethrel (2-chloroethyl phosphonic acid) treatment for 48 hours at 10^{o}C. Besides, androgenic response of rice anthers was enhanced if the inflorescences were pre-treated with ethrel. Similarly, Wang *et al*. (1979) reported positive effect of ethephon on mitotic activity of microspores.

2.2.5 *Culture media*

Haploid plants can be produced either through embryo formation (Guha *et al*., 1970) or callusing (Niizeki *et al*., 1968) by varying media composition. N6, MS and SK1 solid media are in vogue used for anther culture and the former being more potent than two later media (Siddique, 2015) and even compared to MO19, Blayde's, Heh 5, MSN1, SK 8 and R 2 in callus induction (Lenka *et al*., 1994; Chu, 1982; Raina *et al*., 1997) and green plant regeneration (Mishra *et al*., 2011). Frequency of callus formation were better in N6 medium as compared to MS medium (11.9% and 7.95%, respectively) (Thuan *et al*., 2001). Lentini *et al*. (1995) favored liquid media for callus induction as it provides greater access to nutrients and hormones and there are chances of more rapid dispersion of toxic substances (Raina, 1997). Choice of media proves to be genotype-specific (Talebi *et al*., 2007; Ratheika *et al*., 2007). Khanna and Raina(1998) indicated significant genotype x culture media interaction for regeneration response in three indica rice cultivars. N6 media respond better to anther culture in japonica than indica (Lentini *et al*., 1995). Kaushal *et al*. (2014b) reported highest callus induction, green plant regeneration and least albino plant development of 40.64%, 40.93% and 3.72% respectively in He-2 medium.

Higher doses of nitrogen, phosphorus and potassium in the media led to better anther culture response in indica rice (Silva, 2010). Generally ammonium nitrogen ($NH4^{+}$) is required in low amount for anther culture in cereal. N6 medium being high in $NO3^{-}$ and low in ($NH4^{+}$) has proved to be very efficient for japonica rice anther culture (Grimmes *et al*., 1990). Sucrose and iron are crucial for pollen embryo development (Nitsch, 1972) and chelated form of iron (Fe-EDTA) is more effective than ferric citrates (Rashid *et al*., 1973). Maltose showed better callusing and

regeneration response than sucrose. Besides, higher ratio of green plantlets to albinos in both japonica and indica types was realized due to mannitol as carbon source(Javed *et al*., 2007; Bishnoi *et al*., 2000; Pande *et al*., 1999; Trejo-Tapia *et al*., 2002). Alternatively, ficoll- a synthetic polymer of sucrose could be used to increase the surface density and improve the ratio of green to albino plantlets (Lashermes, 1992) and to maintain optimum osmotic pressure. Gelrite is another gelling agent found to be the most effective one(Lee *et al*., 1995). Addition of organic additives (yeast extracts@100 mg/l, casein hydrolysate @500mg/l, coconut water 5-10%) etc) to N6 further enhanced androgenic callus induction in indica rice varieties (Roy *et al*., 2005). Besides, requirement of amino acids e.g., glutamine and alanine is realized in indica rice for callus formation and green plant regeneration (Ogawa *et al*., 1995). $AgNO_3$ supplementation can induce callusing response and promote the regeneration of green plants (Sah, 2008).

2.2.6 *Hormonal requirement*

2,4-D and NAA alone or with kinetin in the culture medium seem to be the major determinants for embryogenic callusing from rice anthers(Trejo-Tapia *et al*., 2002; Herath *et al*., 2008; Lal *et al*., 2014; Mukherjee *et al*., 2015). Neither 2,4-D nor NAA alone in the media can support regeneration, but the use of cytokinins like Kn and BAP with NAA are required for regeneration. A combination of 0.5ppm IAA (Indole acetic acid) and 2.0ppm facilitates germination of androgenic embryos. IAA and NAA may induce direct androgenesis, while 2,4-D promotes callus induction and rapid cell proliferation (Bal *et al*., 1993). Lower concentration of BAP enhances microtillering from androgenic plantlets (Roy *et al*., 2011) and NAA promotes the formation of roots. 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 supplemented with 2,4-D (0.5mg/L)+ NAA (1.0mg/L)+ BAP (0.5mg/L). Nirouli and Bimb (2009) reported higher callus induction frequency in N6 medium with 2,4-D(2.5mg/l) + 0.5mg/l Kn than N6 +NAA (4mg/l) + Kn(0.5mg/l); but reverse was the case for green plant regeneration. Xa and Lang (2011) reported 5.13% to 9.27% callus induction and 6.17% to 14% regeneration from four crosses in MS medium with combination 1mg/L BA + 2mg/L Kinetin + 3% sucrose. Besides, MS medium with 10% coconut milk (CM) and addition of Kn (0.5mg/1), BAP (2 mg/1) and NAA (1 mg/1) at ratio of 1:4:2 gave consistently high frequency of plantlet

regeneration from anther derived calli of a wide range of genotypes (Rout *et al*., 2001).

Embryo formation (10.8%) was reported to be markedly increased by ABA (abscisic acid) in the callus induction media or its treatment during the cold pre-treatment period (Sohn, 1996). Ethylene is also produced in plant cell due to the presence of auxin (Yang *et al*., 1984), sucrose (Zhou *et al*., 1991) or calcium (Hepler, 2005) in the callus induction medium. Profused callusing may be due to inhibitory effect of endogenously produced ethylene from excised anthers (Ghamemi *et al*., 1994). Polyamines (Dewi *et al*., 2008) and $AgNO_3$ (Lentini *et al*., 1995) also affect anther culture response in rice through inhibition of ethylene synthesis. Putrescine application to the callus medium also increases the frequency of green/albino regeneration frequency both in japonica and indica variety.

2.2.7 *Culture condition*

Anther derived callus was observed in Azucena rice variety (6.66%) while no callusing was found in Budda variety when the cultures were exposed to dark at 23±2°C (Lal *et al*., 2014). Highest callus induction was observed from the anther culture of rice variety Rajalaxmi and Ajay by keeping the culture at 25 ± 10^0C for 3-4weeks (Mishra *et al.,* 2011) and from anthers of six F_1 hybrids within 60days and highest regeneration frequency was obtained by keeping at 18h photoperiod with a light intensity of 27 µmol $m^{-2}s^{-1}$ at 25±1°C (Lee *et al*., 2003a) which was also observed in japonica variety (Mankeumbyeo, recurrent parent) and a recalcitrant indica variety (Ranta Emas, donor parent) at the same culture condition (Lee *et al*., 2004).

Culturing of anthers of PT-1, KDML-105, and HJ rice variety at 25±2°C in darkness for 6 weeks (Cha-um *et al*., 2009). Highest callus induction frequency was obtained in anther culture of Japonica × Indica, Indica × Japonica hybrids (Trejo-Tapia *et al.*, 2002) at 25±2°C for 8 weeks. Androgenic callus induction as well as green plantlet regeneration was observed by keeping anther culture of five indica rice genotypes (IR 72, Mansarovar, Taraori Basmati, Pusa Basmati and Karnal local 95) (Roy *et al*., 2005) and indica rice genotypes (GR 11 and Gurjari) in dark at 25±2°C (Patel et al., 2014). Callus induction frequency and regeneration efficiency at $30^oC/20^oC$ day/night temperature in the dark for 4 weeks was increased 1.4 times and more than double respectively than when kept at 25°C (Okamoto *et al*., 2001). Initial incubation in dark at 26±2°C for 6 weeks followed by 12 h photoperiod for another 2

weeks resulted highest callus induction and plant regeneration response in six indica (HKR120, HKR 86-3, HKR86-217, PR106, Gobind and CH 2 double dwarf) and two Basmati rice (Basmati 370, Taraori Basmati) varieties and 14 heterotic indica Basmati F_1 hybrids (Bishnoi *et al*., 2000). Callus induction was also observed by incubating at 27^oC, but regeneration ability was lost after 11days (Chen *et al*., 2001). In contrast, incubation at above temperature was satisfactory in an interspecific hybrid (*Oryza sativa* L.x *O. rufipogon*) for callus induction in dark and for plantlet regeneration in16h photoperiod (2000 lux) (Mandal *et al*., 1997). However, callus induction frequency (0.75 to 5.15%) was found from anthers of two indica elite lines (MRP5401 and BR29) and one japonica variety (Taipei 309) with exposure in white light for 16h at 28 ± 2^0C until shoots development in about 3-6 weeks (Bhatacharya *et al*., 2014).

2.3 Ploidy status of anther derived calli and plantlets

Various morphological attributes are used to verify the ploidy status as suggested by Sah and Niroula (2007). Haploids and DH can be identified by comparing plant morphology such as plant height, leaf size, flower morphology, plant vigor and fertility, Haploid plants show reduced plant vigor with degenerated flowers or anthers and are mostly sterile or show greatly reduced fertility, whereas diploid individuals resemble the donor plant and are often characterized by normal flower and pollen development (De *et al.*, 1987). However, this method is not 100% full proof. Cytological analysis of anther/pollen culture can be used to unfold the factor(s) controlling pollen embryogenesis of higher plants. Study of meiotic behaviour of haploids provides a status of chromosome duplication. The principle of androgenesis is to arrest the development of male gametophytes (pollen grains) and to force them to follow sporopytic (somatic) pathway (Datta, 2005). Under certain culture condition in anther culture, the pollen grain(haploid) induce, divide and then double the chromosome number while reversing the process of gametophyte(early to mid-uninucleate) to sporophytic stage. The ploidy status of regenerated plants can be confirmed by counting chromosome number in root tip and in pollen mother cells following usual acetocarmine staining technique (Maluszynska, 2003). Seventy percent of the regenerated plants are normally haploids while, rest accounts in vitro euploids (including spontaneous diploids) and aneuploid. The ploidy level in plants can be also estimated by chloroplast count in guard cells or chromosome count of root tip cells (5-8 in haploids, 10-12 in diploids and 15-18 in triploids and still more in

case of higher order of ploidy levels). However, recently measuring the C-value (amount of DNA in the unreplicated gametic nucleus) using flow cytometry seems to be a suitable approach (Ochatt *et al*., 2009).

2.4 Production of double haploids

Doubled haploid lines derived from anther culture of F_1 hybrids are promising tools to develop plant cultivars. Colchicine (a mitotic inhibitor) treatment is *in vogue* used to induce chromosome doubling by inhibiting anaphase movement in crop plants (Lee *et al*., 2003a). Colchicines treatment of apical meristem of anther derived haploid plants produce doubled haploid cells in small sectors which normally produce DH seeds. Microspore derived calli often show aneuploids, dihaploids and polyploids (Lee *et al*., 2004). Occurrence of mixoploids and polyploids resulting chimeric plants and low seed set is prevelent in colchicine treatment, hence normally avoided at whole plant level in rice. However, the application of colchicine during early stages of androgenesis could alleviate above problems (Castillo *et al*., 2009) and increase the frequency of DH development (Alemanno *et al*., 1994) in shortest time. Supplementation of 0.2-0.5g/L colchicine for 24-48h incubation results viable double haploid embryo-like structure (ELS) which on transfer to colchicine-free regeneration medium could induce as high as 65-70% viable DH plantlets and it was twice compared to spontaneous doubling (Alemanno *et al*., 1994; Premvaranon *et al*., 2011). Alternatively, the anther culture derived haploid plants may be emersed in 0.1% colchicine and 2% dimethylsuphoxide(DMSO) solution for 4-5 hours (Zapata-Arias, 2003) or colchicine(1.25mM) and oryzalin(25µM) solution for 12 hours (Chen *et al*., 2001) to obtain mirror image of the haploid set of chromosomes in DH lines. In rice, the haploid set of chromosomes in microspores also becomes spontaneously doubled under suitable culture conditions. Germana (2011) reported higher percentage (50-60%) of spontaneous chromosome doubling (endoreduplication) in culture. Lee *et al*. (2003b) recovered a maximum of 60% spontaneous DH green plantlets through mutagenesis in japonica rice. Besides, Chen *et al*. (2001) demonstrated that colchicines (30mg/L) have significant stimulating effect on regeneration of higher frequency of green DH mutant plantlets from anther culture of rice varieties. Thus, colchicines treatment may be omitted for production of double haploids in rice.

Jones et al. (Jones *et al*., 1996) was successful to recover DH lines from hybrids *O. sativa* x *O. glaberrima*. Bishnoi *et al*. (2000) produced higher number of

DH lines in indica rice (var. Gobind, HKR120, Basmati 370, and Taraori Basmati) and an indica-basmati hybrid via colchicine treatment (0.1%, w/v). Sasmita (2009) observed more than 90% cases of anther culture, homozygous and stable plants progeny were observed from generation to generation. However, Bhattacharya *et al*. (2014) advocated chromosome counting combined with segregation analysis can distinguish diploids, haploids and double haploids plants.

2.5 Genetic basis of anther culture response

Genetic control of microspore response to *in vitro* culture in rice has been studied by a number of workers (Guha-Mukherjee, 1973; Aruna *et al*., 1988; Zhang *et al*., 1993; Yan *et al*., 1996; Roy *et al*., 2005). It is largely species and genotype-specific (Gueye *et al*., 2010; Dash *et al*., 2014). *O. glaberrima* responds more to callus induction and plantlet regeneration than *Oryza sativa* genotypes. All *O. glaberrima* genotypes regenerated plants and few genotypes of *O. sativa* subgroup japonica only produced plants, whereas, indica was recalcitrant to plantlet regeneration. Indica rice is known to have a recalcitrant genetic background showing low anther culture response (1.2% callus induction), whereas japonica cultivars had more than 20-fold responsive (28.1%) to microspore embryogenesis (Grewal *et al*., 2011).

Diverse genotype specificity of anther culture do exist within indica subspecies (He *et al*., 2006; Shahnewaz *et al*., 2004; Ratheika *et al*., 2007; Talebi *et al*., 2007). Temperate rice (hill rice) cultivars are *in vogue* more responsive to anther culture than tropical rice (Niroula *et al*., 2009). Anther culture ability is reported to be decreased in the order of japonica/japonica F_1 > japonica > indica/japonica F_1 > indica/indica F_1 > indica (Yan *et al*., 1996). IR 43 produced maximum embryiods (16.13%) and green plantlets (11.88%) followed by BRRI Dhan 33, IR 54, Jaya and BR3 (khatun *et al*., 2012). Indica x indica hybrids produce more of albino plantlets compared to Indica x Japonica hybrids (Rangaswamy *et al*., 1992). However, CRMS31B x CRMS24B among 4 inter-varietal crosses, was highly responsive for callus induction (37.83 %) as well as shoot regeneration (41.09 %) and green plant regeneration (15.00 %).

Genes encoding certain enzymes are essential for metabolism of differentiating cells, tissues and organs. Calli with higher levels of the peroxidase enzyme has been reported to display a greater regeneration potential than those with

lower levels of the enzyme producing predominantly albinos (Subhadra *et al*., 1998). Induction of sporophytic haploidy in rice anther culture is in vogue controlled by haploid (gametophytic) inhibitor gene 'hap' (Kasha *et al*., 1983) 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*., 2014b). Japonica/indica crosses usually result partial sterility due to interaction between the indica allele S-5i and japonica allele S-5j in chromosome 6. This causes partial abortion of female gamete containing S-5j. A recent report shows that the "indica T 23" rice genetic stock with wide compatible allele S-5n (in chromosome 6) respond well to anther culture (Nguyen *et al*., 2016). Dular -a indica variety has also the allele S-5n at S-5 locus which is widely compartible with both S-5i and S-5j. However, many often segregation distortion (SD) at S-5 locus is reported and it may be due to preferential selection of gametes with S-5i indica allele for androgenesis (Yang *et al*., 2012). Such genetic stock is being attempted for introgression of anther culture response into the indica genetic background by back crossing.

Callus induction from anthers and plant regeneration from the induced callus in rice are the two independent traits displaying quantitative inheritance (He *et al*. 2006; Bagheri *et al*., 2008; Miah *et al*., 1985; Quimio *et al*., 1990; Yamagishi *et al*., 1998). Genetic analysis could be performed on haploid population to establish inheritance patterns. Additive effects seems to be more important than dominant effects of the genes concerned for callus induction while preponderance of dominant effects is reported for regeneration response (He *et al*. 2006). Quimo and Zapata (1990) suggested predominance of additive effects controlling both characters with japonica cultivars having higher general combining ability for green plant regeneration. However, callus induction is reported to be mainly controlled by gametic additive effects than maternal effects, while reverse is the case for green plantlet regeneration in anther culture (Yan *et al.*, 1996). Selection of parents with high GCA and most promising crosses revealing high SCA may pave the way for recovery of segregants with improved anther culture response. Anther culture response is reported to follow recessive inheritance (Miah *et al*., 1985) conditioned by

block of genes (QTLs) on two specific chromosomal regions and japonica appear to be a good combiner for callus induction. A QTL on chromosome 1 control callus formation and another on chromosome 10 determine the balance between albino/green plant regeneration ability (Yamagishi *et al*., 1998). However, He *et al*. (1998) identified five quantitative trait loci (QTL) on chromosomes 6, 7, 8, 10 and 12 responsible for callus induction and two QTL on chromosomes 1 and 9 for green plantlet regeneration. Besides, they identified a major QTL for albino plant differentiation on chromosome 9. Kwon *et al*. (2002a, 2002b) mapped two candidate loci (QTLs) for green plantlet regeneration on chromosome 3 and 10 and identified molecular markers that co-segregate with these genes. Molecular markers such as RAPD (Afza *et al*., 2001), AFLP (Guzy- Wrobelska *et al*., 2003) and SSR (Semagn *et al*., 2007; Lee *et al*., 2014) have proven to be a powerful tool for characterization of DH lines. Grewal *et al*. (2011) used a set 124 DH lines for SSR marker analysis generated from the japonica cultivar (IR69428) ×indica variety (IR64) that showed 1:1 ratio of indica and japonica alleles. Homozygosity was detected for all the marker loci in 124 DH lines and the genes for anther culturability were shown to be partially dominant. Besides, Hemaprabha *et al*. (2013) established genetic differences among anther culture-derived stable breeding lines using RAPD and ISSR markers. Marker-assisted selection (MAS) strategy can improve anther culture ability in indica rice (Bolibok *et al*., 2006). Recent studies have identified several genes that are specifically expressed in calli with good regeneration ability and some have been cloned (Silva, 2010). 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).

2.6 Double haploid breeding

Anther culture is a useful biotechnological tool applied in plant breeding. Creation of genetic variability is the essential step in any crop improvement programme. Breeders often resorted to hybridization or mutation breeding and even genetic transformation for genetic improvement of specific trait(s). Hybrids being possess good broad genetic backgrounds than their parents, breeding them through double haploid production is an important method. Double haploid breeding provides fertile segregants with fixed derived character combination that might otherwise disappear in the course of an extended series of segregating generations in

conventional breeding methods. Variation in DH lines from F_1 hybrids is due to recovery of rare gene combinations (following meiosis) even in a small population size (Dhaliwal *et al*., 1997) of 200 double haploid plants. However, plants derived from multiple shoots of a single callus show high phenotypic similarity than those derived from different calli (Ayres, 1995). The regenerants may show heterozygosity due to anther wall somatic tissues (Morrison *et al*., 1988) and a wide range of gametoclonal variation in addition to biochemical and chromosomal changes (Narasimman *et al*., 1993). Such genetic variation among DH lines can be harnessed for improvement of agronomic characters and the production of new cultivars and breeding lines (Wernsman, 1992).

Sterility problem in interspecific cross (*O.sativa* x *O.glaberrima*) was made possible to overcome by double haploid approach (Enriquez *et al*., 2000). A few DH lines derived from anther culture of hybrids of genetically diverse parents could reach the heterotic level of the hybrid (Ba bong *et al*., 1995) which further pinpoints the prospect of anther culture technique in rice. DH lines with earliness enhanced biomass production, high panicle density and increased yield potential with resistance to pest and grain quality could be produced from anther culture of different crosses (Zhu *et al*., 1993; Rangaswamy *et al*., 1992). DH lines from cv. Calrose 76 showed variation with improved agronomic characters e.g., seed number and size, panicle size, plant height and tiller number and improved protein content and higher yields (Schaeffer, 1982). Roy & Mandal (2005) reported high genetic variability for filled grains per panicle, grains yield per plant and number of panicles per plant among androgenic plantlets derived from scented indica rice cv. Karnal local 95, suggesting scope of haploid breeding for higher seed yield. Xa and Lang (2011) developed 22 outstanding DHLs for high grain quality through anther culture. Bhattacharya *et al*. (2014) confirmed 98 lines to be DH out of 232 lines recovered from Mahyco Hybrid MRP5401 which had dwarf height, early to late flowering and high yield.

Development of improved DHs for salt tolerance (Lee et al., 2003a), submergence tolerance (Mandal *et al*., 1997) and bacterial blight resistance (Sengsai *et al*., 2007) have been reported. Mohiuddin *et al*. (2014) recovered genetically uniform dwarf DHs from anther culture of an advance breeding line BR802-78-2-1-1. Few of these DHs revealed high fertility status of spikelet with long-bold and long-slender grain. Anitha and Reddy (1997) could successfully recover salt tolerant plantlets from anther culture derived calli of Pokkali and Korgut subjected to 100mM

NaCl stress. Fertile DH plantlet regeneration was also reported in a salt susceptible × salt tolerant rice hybrid (Chowdhury *et al*., 2001). DH lines produced from anther culture have many advantages like improved grain quality, resistance to diseases, tolerance to biotic and abiotic stress, superior performance for some agronomic traits over the parents and/or lines used as checks (Winzeler *et al*., 1987; Mitchell et al., 1992; Courtois, 1993). Mishra *et al*. (2015) studied physico-chemical characteristics and cooking quality traits were studied on twenty recombinant doubled haploid lines derived from rice hybrid Ajay and Rajlaxmi. Eight derivatives from each hybrid possess all the grain quality characteristics at desired levels and some were better than their respective parents in terms of both quality and uniformity. Xa and Lang (2011) recovered 133 DH lines out of which 22 outstanding DH lines were selected for yield and grain quality. Purwoko *et al*. (2010) recovered 92 doubled haploid lines from 13 crosses out of which 24 lines had seed yield more than 26g per hill (transplanted single seedling per hill) with tolerance to biotic and abiotic stresses.

More than 200 crop varieties have been developed worldwide by double haploid approach (Thomas *et al*., 2003; Zapta-Arias, 2003) among which more than 40 are rice varieties (Table 1). Senadhira *et al*. (Senadhira *et al*., 2002) developed first salt tolerant rice cultivar through indica/indica anther culture. DH breeding was also successful for developing new cultivars of rapeseed (‘Maris Haplona’), wheat (‘Emerson’ and ‘BRS 328’) and barley (‘Mingo’) (Scheeren *et al*., 2014). ‘Dama’ is the first Hungarian rice variety developed by haploid somaclone breeding in 1992 (Heszky *et al*., 1992). In India, Satyakrishna (CR Dhan 10) and Phalguni (CR Dhan 801) are the first released varieties from DH lines. Guan 18 and Gan Xhao Xian No. 11 are the outcome of anther culture in china which mature within 105days and possess high yield potential (5.6-6.0t/ha). Besides, several rice varieties have been developed by anther culture in China (Tanfeng 1, Bua Yu 2, Hua Yu 1, Hua 03, Xin Xiu, Xhongua 8, Ta Be 78), Argentina (Patei and Moccoi).

Table 1. List of rice varieties released using double haploid breeding

Country	Rice variety	Trait(s)	Ref.
China	Guan 18, Gan Xhao Xian No. 11	Early maturity; good quality and disease resistance	Zhu *et al*., 1990
South Korea	Hwacheongbyeo, Joryeongbyeo, Hwajinbyeo	Brown plant hopper, stripe virus, blast and bacterial blight resistance	Lee *et al*., 1989
India	CR Dhan 801, Phalguni	Leaf blast, gall midge resistance	CRRI Annual Report 2007-2008
India	CR Dhan 10, Satyakrishna	Resistant to neck blast, sheath rot	CRRI Annual Report 2009-2010
China	Huayu 15	Lodging and diseases resistance; good grain quality	Shouyi *et al*., 1991
Republic of Korea	PSBRc 50 Bycol (IR 51500-AC-11-1)	Salt tolerance	Senadhira *et al*., 1996
Philippines	PSBRc 50 Bycol (IR51500AC11-1)	Salt tolerance	Senadhira *et al*., 2002
India	Janka	Drought tolerance; good grain quality	Pauk *et al*., 2009
India	Abel	Cold tolerance at early stage	Pauk *et al*., 2009
Japan	Joiku N 394, Hirohikari, Hirohonami, AC. No.1, Kibinohana	Cold tolerance and good taste	Singh, 1998
China	Huayu I, Huayu II, Xin Xiu, Late Keng 959, Tunghua 1, Tunghua 2, Tunghua 3, Zhonghua 8, Zhonghua 9, Hua 03, Huahanzao, Huajian 7902, Tanghuo 2, Shanhua 7706, Huahanzao 77001,Noll	High yield, superior grain quality; blast and bacterial blight resistance	Hu *et al*., 1984; Chen, 1986; Loo *et al*., 1986; Yang *et al*., 1989
Hungary	Dama	High yield, resistant to *pyricularia*,good cooking quality	Heszky *et al*., 1992
China	Milyang 90	Good grain quality; brown plant hopper and stripe virus resistance	Chung, 1987
India	Parag 401	Superior grain quality and resistant to iron chorosis	Patil, 1997
China	Hua 03	High protein content (13.7%)	Yang *et al*., 1989
India	Risabell	High milling, good cooking quality and resistant to blast	Pauk *et al*., 2009

Anther culture derived DH populations provide a link between conventional plant breeding and genomics. DH populations are quite useful for chromosome mapping (Forster *et al*., 2005) and genetic analysis of QTLs (Lu *et al*., 1996). Double haploid lines being permanent fixed genotypes (Semagn *et al*., 2006); minimize the environment component of variation and allow accurate measurement of quantitative traits. Such permanent mapping population offers more advantage over segregating F_2, F_3, BC_2, BC_3 populations and is often preferred over near isogenic lines(NILs), back cross inbred lines(BILs), recombinant inbred lines(RILs), multiparent recombinant inbred lines (MPRILs), chromosomal segment substitution lines (CSSLs), multi-parent advanced generation intercrosses (MAGIC), nested association mapping (NAM) populations which require long time to develop (Singh *et al*., 2015). In DH populations, the Identification of DNA markers seems to be much reliable as the gene segregates in the ratio of 1:1(dominant homozygous : recessive homozygous) and most intermediate phenotypic expressions due to heterozygosity are excluded.

Anther derived DH populations of rice have been used in QTL mapping of rice seed dormancy (Guo *et al.*, 2004), grain quality (Ren *et al*., 2016; Huang *et al*., 2012), cooking quality (Tian *et al*., 2005), seed yield (Park *et al*., 2014) and resistance to brown plant hopper (Hu *et al*, 2016), leaf folder (Rao *et al*., 2010), blast (Jichen *et al*., 2004), drought (Xu *et al*., 2001) and salt stress (Fan *et al.*, 2015). Besides, validation of quantitative trait loci associated with reproductive-growth traits and grain yield under drought stress in a doubled haploid population of rice (*Oryza sativa* L.) was studied by Sellamuthu *et al.* (2011). Double haploid (DH) line population derived from the cross CT9993-5-10-1-M/IR62266-42-6-2 is tested for the QTL mapping of drought resistance in rice. DH breeding can be also useful for functional genomics studies and fixing a transgene while simultaneously removing unwanted selectable marker gene (Kapusi *et al*., 2013). Jiang *et al*. (2004) elucidated the genetic basis of stay-green in a population of doubled haploid lines derived from an indica x japonica cross. The development of anther culture may be also used as a system for in vitro mutant selection (Chaleff *et al*., 1982).

2.7 Molecular markers associated with drought tolerance:

Rice is an excellent model plant for genome analysis as its genome size is one of the smallest among the major cereal crops and only two or five times larger than that of Arabidopsis. The physical length corresponding to the unit genetic distance is

also small (100-300kb) comparable to that of Arabidopsis calculated from genome size and the map length. The molecular techniques have the potential to complement the conventional breeding as these ensure genetic identity, authenticity and for assessment of genetic diversity and screening of mutants/breeding materials including double haploids in rice. Molecular markers can aid selection for target alleles in individual plants and to minimize linkage drag around the target gene.

Polymorphic fragments between two parents of the F_1 plants were identified and their segregation patterns in anther culture derived plants can be used to elucidate the origin of those plants and to determine the degree of independence of plants regenerated from the same callus.

Reddy *et al.* (2009) determined genetic relationship among 12 rice varieties including nine tolerant to drought, flood or salinity using ISSR markers. The results revealed that ISSR markers associated with (GA)8 YG delineated the three groups of stress tolerant varieties from each other and can be used to identify genes/new alleles associated with three abiotic stresses in rice germplasm. Nasr *et al.* (2009) attempted to detect some ISSR-PCR markers linked to drought tolerance in some local wheat genotypes, using bulked segregants analysis. Total soluble proteins and peroxidase isozymes electrophoresis patterns can also be monitored in relation to drought stress. Kanagaraj *et al*. (2010) studied to identify markers linked to drought resistance using 23 recombinant inbred lines of IR 20 /Nootripathu, two indica ecotypes with extreme drought resistance. The parents were screened for polymorphism using 1206 rice microsatellite primer pairs. Out 134 SSR polymorphic primers between parents, three primers showed polymorphism between bulks. These three primers co-segregated among the individual RI lines constituting the respective bulks.

Youssef *et al.* (2010) identified two amplified fragments of about 315bp and 505bp using a ISSR primer HP 15 in the genomic DNA of the drought tolerant lines but these fragments were absent in the sensitive cultivars. Such fragments /bands can be considered as positive drought tolerant markers. Lin *et al.* (2017) raised a F_2 mapping population from the cross between Taichung 189 (japonica type), a local drought susceptible rice, and Milyang 23 (indica type), a drought tolerant line. A total of 525 SSR DNA markers were used to screen polymorphisms between parents. There were 121 SSR DNA markers showed polymorphisms and were mapped on the F_2 population. A total of 4 SSR DNA markers, RM 518(dis4.1), RM 72(lr8.1), RM

228 (lr10.1), and 20A(lr12.1) on chromosomes 4, 8, 10, and 12 were associated with drought tolerance (based on leaf rolling).

Lang and Buu (2008) evaluated two hundred twenty nine (BC_2F_2) plants derived from a cross between OM1490 / WAB880-1-38-18-20-P1-HB for drought tolerance. RM 201 was identified to be an important molecular marker (on chromosome 9) for drought tolerance. Similarly, Aboulila (2015) identified two molecular markers RM201 marker and RM451 to play an important role in drought response in rice genotypes. Besides, out of 343 rice microsatellites markers genotyped, RM8085 was reported to be co-segregated among the RI lines constituting the respective bulks (resistant and susceptible) (Arvindkumar *et al*., 2011). RM8085 was mapped in the middle of the QTL region on chromosome 1 previously identified in these RI lines thus reducing the QTL interval from 7.9 to 3.8 cM. Further, the study showed that the region, RM212–RM302–RM8085–RM3825 on chromosome 1, harbours large effect QTLs for drought-resistance traits across several genetic backgrounds in rice. Thus, the QTL may be useful for drought resistance improvement in rice through MAS and map-based cloning. Kumar *et al.* (2005) identified out of seven polymorphic primers between the bulks (resistant and susceptible), two primers - RM223 and RM263 co-segregated in all the individual rice accessions constituting the bulks. These closely linked SSR markers associated with drought resistance may be useful in marker-assisted selection (MAS) for genetic improvement of rainfed rice.

MATERIALS AND METHODS

3.1 PLANT MATERIALS

F_1 plants from a cross Khandagiri (an improved high yielding upland rice variety) x Dular (a drought tolerant local land race) were used in this study. The cross combination was purposefully chosen from a set of crosses on the basis of segregation potential. Pure seeds of both the parents were obtained from Economic Botany- I, Department of Plant Breeding & Genetics, OUAT, Bhubaneswar. 30-day old F_1 seedlings were transplanted using standard agronomic practices. Staggard planting of F_1 plants was done at 1-week interval for five sowing dates to avail anthers required for in vitro culture.

3.2 ANTHER CULTURE OF F_1

3.2.1 Preparation of Plant Materials

Boots from primary tillers were collected at around 8.00am 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 reach 5-8cm. This usually corresponds to the mid- to late uni-nucleate stage of pollen development in anthers of the central spikelets. The boots were wrapped with tissue paper followed by alluminium foil and placed inside the refrigerator at 4^oC for one week in dark. After cold treatment, spikelets from middle portion (Alejar *et al*., 1995) of the panicles were disinfected with aqueous mercuric chloride (0.1%, w/v) for 1minute under aseptic condition followed by washing (x 5) with sterile distilled water and excess water was soaked with sterile blotting paper. Before incubation of anthers in the callus induction medium, microspore developmental stage in central spikelets was checked by acetocarmine staining under light microscope.

For cytological study, young spikelets at the suitable stage were fixed in acetic acid-alcohol (1:3) mixture for 24 h, and pollen mother cells at the meiotic division stage were stained using smearing technique with 1% aceto-carmine stain solution. Panicle having mid and late microspore stage are *in vogue* suitable for anther culture (Rukmani *et al*., 2011). As described by Lentini (Lentini *et al*., 1997), 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 expose anthers.

3.2.2 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 & Skoog, 1962) and a formulated callus induction medium (CIM: a modification of MS) with different hormone recipes (2,4-D, Kn and NAA) were used for callus induction(Table 1). Besides, a regeneration medium (RM) with Kn, BAP and NAA at different concentration was also prepared as a modification of MS medium to suit plantlet regeneration from anther derived calli. 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 minutes. 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 $25\pm1^{o}C$ and RH 65% for callus induction. Calli of approximately 0.5cm diameter are 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.

Table 2. Composition of four minimal media used for anther culture

Components	N6	SK3	MS	CIM	RM
NH_4NO_3	-	-	1,650.0	1,000.0	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_4, $7H_2O$	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	5.7

N6: Chu et al. (1978), SK3 (a modification of N6): Chen et al.(1978), MS: Murashige T & Skoog F (1962), CIM(a modification of MS) : Callus Induction Medium, RM: Regeneration medium

3.2.3 Plant establishment

The plantlets regenerated through organogenesis and somatic embryogenesis were initially transferred to half-strength MS basal liquid medium for one week before transfer to pots filled with soil mixture of peatmoss: perlite 2:1 (v/v). Thereafter, plantlets were successfully acclimatized and cultivated in glasshouse under partial shade. Altogether 55 putative DHs out of total 123 anther derived plantlets came to normal flowering and produced filled grains. Such *in vitro* derived DH plants were harvested individually to raise DH lines (in late Rabi 2016-17) to be used as a mapping population in future study. Besides, a part of seeds harvested from individual anther culture derived DH plants was used to assess their status for drought tolerance.

3.3 SELECTION OF DHs FOR DROUGHT TOLERANCE

3.3.1 Artificial screening using PEG

Two sets of genotypes comprising 55 DHs + 2 parents (Khandagiri and Dular) were germinated in plastic cups and seedlings were raised using half-strength

Yoshida's nutrient solution. After one week, artificial drought stress was imposed in one set of genotype by replacing half-strength Yoshida's nutrient solution containing 20M PEG_{6000}, while the other set of genotype serving as control, was continued with normal nutrient solution. Three days later, PEG concentration was raised to 30M in the nutrient solution and maintained for another one week. The nutrient solution with PEG was changed on alternate days. The pH of both the stress treatment and control was adjusted to 5.0 by adding 0.1N HCl or NaOH solution every 24 hours. Drought tolerance score based on leaf drying (0-9 scale), root length and shoot growth of each DH were recorded after above period of stress treatment.

3.3.2 Screening in net house (pot culture)

30-days old seedlings of each DH were transplanted in earthen pots lined with polythene and drought stress was imposed for three consecutive days at vegetative (50 DAS) and panicle initiation stage. Drought tolerance score after 3days of water stress(no watering) and drought recovery score (0-9scale) after rewatering of each DH were recorded.

3.3.3 Marker aided selection

Molecular profiling of genomic DNA was carried out using a gene specific SSR primer for both the parents to assess parental polymorphism and to assist selection of drought resistant plants in a set of double haploid population.

3. 3.3.1 *Isolation of DNA*

Genomic DNA of single anther culture derived plants (DHs) was isolated from tender young leaves at seedling stage on the same day of collection using standard CTAB method. The plant materials were homogenized in liquid nitrogen and extracted with extraction buffer(100mM Tris-HCl pH 8.0, 20mM EDTA, 2% CTAB, 1.4M NaCl, 0.4% β-ME and 2% PVP) at 65^{o}C for one hour with occasional shaking and an equivalent volume of phenol-chloroform-isoamyl alcohol (25:24:1) mixture was added and centrifuged at 1000rpm for 15 min. at 4^{o}C. The supernatant was added with 0.6 volume of ice cold absolute ethanol and kept for overnight to precipitate DNA. The intact genomic DNA was hooked out and washed with 70% ethanol 2-3 times and finally re-dissolved in TE buffer (10mM Tris-HCl, pH-8.0 and 1mM EDTA). The DNA was purified by DNase free RNase-A (GeNei) @ 20µg per ml. of DNA extract to remove contaminating RNAs. Finally the DNA was quantified through UV-VIS Nanodrop-2000 spectrophotometer (Thermo Electron Scientific Instruments LLC, USA) at 260nm and the quality of DNA was checked using the

ratio of absorbance at 260nm and 280nm. Each DNA sample was diluted to a working concentration of 10ng/µl for PCR analysis.

3.3.3.2 *PCR analysis*

Each individual genomic DNA sample was primed and amplified using the chromosome 1 major QTL/gene specific primer RM 8085 (F: 5'-TGCGTTTCGATTTCTTTTTA 3' and R: 5' GGAAAGTTGTGTTCTTTGGC 3'). PCR amplification was performed in a reaction volume 25µl containing 1X reaction buffer (10mM Tris HCl, pH 9.0, 1.5mM $MgCl_2$, 50mM KCl, 0.01% gelatin), 2.5mM each of dNTPs, 10ng of the primer pair, 20ng of genomic DNA and 1 unit of Taq polymerase (Genei, Bangalore). DNA amplification was carried out in the Gene Pro Thermocycler (Bioer Tech. Co., Ltd, Japan), programmed for 5min at 95^{o}C for initial denaturation, 35 cycles of 1min at 94^{o}C for denaturation, 1min at 55^{o}C for annealing and 2min at 72^{o}C for synthesis and final extension for 7 min at 72^{o}C followed by storing at 4^{o}C till loading to the agarose gel. The amplified products were loaded in 1.5% agarose gel containing 0.5mg/ml of ethidium bromide and electrophoresed at a constant voltage (50v). PCR conditions were optimized to yield reproducible result. The gels were scanned by gel doc system (Fire Reader-Uvtec, Cambridge, UK) for detection of chromosome 1 gene specific alleles. The size of the amplicons was determined by comparing with the lambda DNA ladder (100bp) with known size (bp) fragments.

3.5 OBSERVATION

3.5.1 Callusing and regeneration response

Approximately, 1500 anthers per treatment (hormone recipe) were assessed for androgenic response. Number of anthers forming calli and those revealed embryo development were recorded and expressed in percentage. Data on % of anthers respond to embryo formation in callus induction medium and follow-up embryogenic plantlet regeneration in RM medium were recorded. Similarly, green plant and albino plant regeneration response were estimated in terms of % of anther response for embryogenic green and albino plant regeneration respectively.

$$\text{Callus induction frequency CIF(\%)} = \frac{\text{Number of calli induced}}{\text{Number of anthers inoculated}} \times 100$$

$$\text{Embryogenic callusing response (ECR\%)} = \frac{\text{Number of embryogenic calli induced}}{\text{Number of anthers inoculated}} \times 100$$

$$\text{Embryogenic regeneration response(ERR\%)} = \frac{\text{Number of embryogenic calli regenerated}}{\text{Number of anthers inoculated}} \times 100$$

3.5.2 Assessment for drought tolerance

Seedling growth: Root length (cm) and shoot growth (cm) of each DH were recorded after ten days of stress treatment (PEG).

Leaf rolling score (LRS): It was scored on 0-9 scale at vegetative stage.

Leaf drying score (LDS): It was scored on 0-9 scale after two weeks of stress treatment (PEG) at seedling stage and under moisture stress at vegetative stage and panicle initiation stage (in pot culture) based on percentage of leaf area dried from tip. **0**: No dried, **1**: < 5%,**2:** 5-10%, **3:** 10-20%, **4:** 20-30%, **5:** 30-40%, **6:** 40-50%, **7:** 50-60%, **8:** 60-90%,**9:** >90%).

Drought recovery score (DRS): It was scored on 0-9 scale after re-watering at vegetative stage.

3.5.3 Assessment of agro-economic traits

Each *in vitro* derived DH plant was harvested individually to raise DH line on plant to row basis in late Rabi 2016-17 following augmented design with both the parents (Khandagiri and Dular) and two popular upland rice varieties as standard checks (Sahbhagidhan and Mandakini). Observations on agro-economic traits were recorded on five randomly selected plants from each DH lines for nine biometric traits except days

to maturity and grain yield which were recorded on plot basis. For 1000-grain weight, observation was taken from random sample of seeds of each plot respectively.

Days to 50% flowering (DF): This was recorded as the period from date of sowing to 50% flowering assessed on plot basis.

Days to maturity (DM): Period from date of sowing to date of physiological maturity.

Plant height (PHT): Height from the base of the plant to the tip of the top most panicle at maturity and expressed in cm.

No. of effective bearing tillers/m^2 (EBT): No of effective ear bearing tillers at maturity counted in a square meter.

Panicle length (PL): Length of panicle from the ciliate base to the tip of the top most panicle of the main culm measured in the centimeter at the time of recording plant height.

Number of grains per panicle(G/P) :Average number of fertile grains per panicle counted from 5 top most panicles(each of 5 randomly selected plants).

1000-grain weight (GW): Weight of 1000 filled grains from random sample of seeds of each plot

Fertility percentage (F%) : The ratio of fertile grains to the total number of the spikelets per panicle expressed in percentage.

Seed yield per ha (SY/P): Weight of cleanly threshed dry seeds (in kilogram) per m^2 was estimated from plot yield and multiplied with 10000. The estimated seed yield is expressed in quintals/ha.

RESULTS AND DISCUSSION

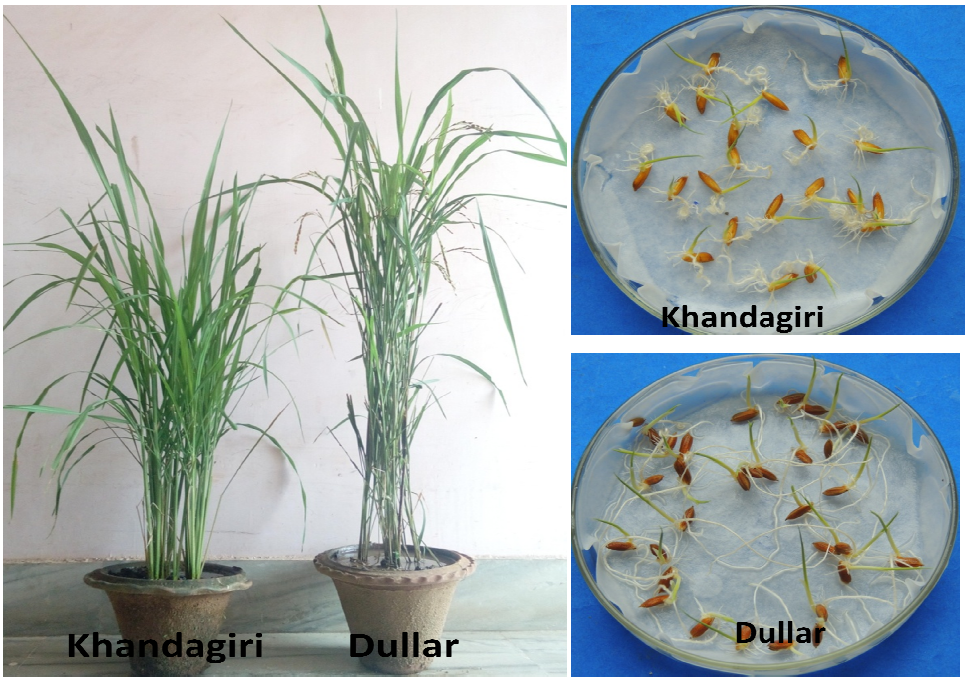
Genetic improvement of rice in India and many other rice growing countries has already achieved yield platuae for medium land irrigated rice ecosystems, but areas pertaining to rainfed rice ecosystems particularly high land situations are constrained with low productivity owing to erratic rainfall distribution. Grain yields are generally as low as 0.5 to 1.5 tonnes/ha in Asia, about 0.5 metric tonnes/ha in Africa and 1 to 4 metric tonnes/ha in South America (Fageria *et al*., 1982). India and Odisha in particular have a vast upland stretches of marginal lands which mostly remain fallow even during rainy season. These areas are often inhabited by socially and politically disadvantaged ethnic minorities. For these populations, food security remains a daily battle. These areas may be brought under cultivation to feed the rural poor.

Drought is a major limitation for rice production in rainfed ecosystems. 11% of the global rice-growing area (IRRI, 2001) is in uplands which face frequent drought leading to drastic reduction in grain yield in rice. Depending on the timing of drought incidence, it may be classified into early stage vegetative drought, late season terminal drought, and intermittent drought. The existing genotypes particularly the short duration rice varieties so far developed are rarely adaptable to pre and post-monsoon drought situation. It has been contemplated that climatic change will compel rice breeders to reorient the breeding strategies and develop high yielding short duration rice varieties to combat the recurrent occurrence of drought in coming years.

4.1 Selection of parents and raising F_1

Odisha is the genetic paradise of rice. Among local land races, Dular is confirmed to have higher level of drought tolerance (Sasmita, 2014), while 'Khandagiri' continues to be a widely adaptable popular high yielding upland rice variety over different agro-climatic conditions of Odisha (Fig 1a). Shamsudin *et al*. (2016) also reported inherent drought tolerance in several traditionally moderate tall landraces such as Azucena, Dular, Rayada and Nagina 22. Therefore, an attempt was undertaken to raise F_1 from a cross Khandagiri x Dular. It is contemplated that such a cross would result a wide genetic variability in the DH population through anther culture.

Hybridization was carried out considering Khandagiri as female parent and Dular as male parent. Dular is characterised by pigmented(black) gynoecium while Khandagiri was known to have greenish white gynoecium. Similarly, Dular retains epiculous pigmentation in matured grain following daugh stage. In contrast, Khandagiri does not contain any epiculous pigmentation. Pigmented gynoecium and epiculous pigmentation on grain in Dular are dominant traits. Hence, plants raised from crossed seeds which revealed both such dominant traits are confirmed to be genuine cross (Fig.1b).



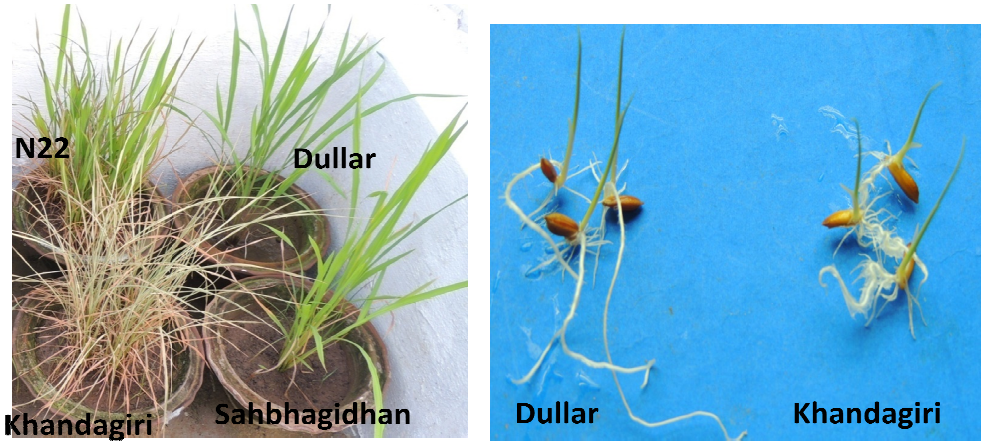


Fig. 1(a). Parent varieties of a cross Khandagiri x Dular

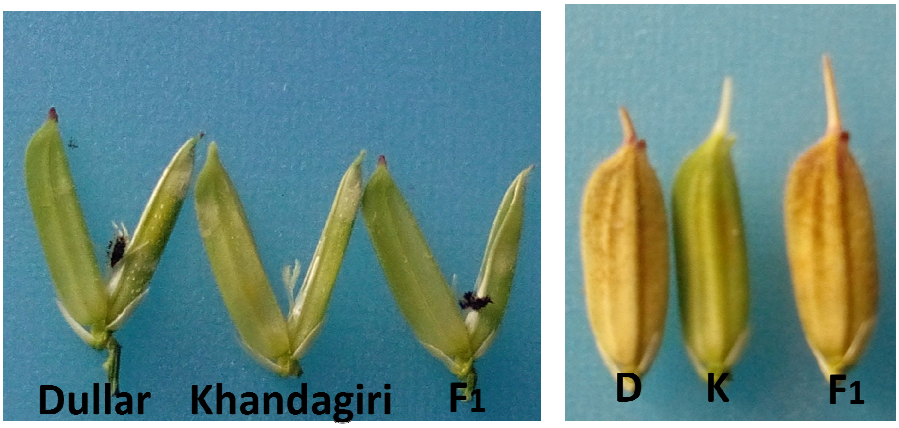


Fig. 1(b). Identification genuine F_1 in a cross Khandagiri x Dular based on dominant markers in F_1

Table 3. Anther culture response of a F_1 cross Khandagiri x Dular for callus induction in different media

Hormone recipe			N6		SK 3		MS		CIM	
2,4-D	Kn	NAA	CIF (%)	ECR (%)	CIF (%)	ECR (%)	CIF (%)	ECR (%)	CIF (%)	ECR (%)
0.0	0.0	2.5	2.2	0.8	12.8	8.0	1.2	0.6	5.5	0.8
0.0	0.5	2.5	5.5	2.0	14.6	10.4	3.0	1.1	6.6	2.8
0.5	1.0	2.5	6.9	4.8	14.8	11.6	12.4	8.4	20.2	16.2
0.5	2.0	3.5	12.8	10.2	20.2	18.3	18.4	10.2	36.5	23.4
1.5	0.0	0.0	8.8	8.0	12.4	10.2	10.2	8.5	20.0	18.5
1.5	0.5	0.0	14.3	12.8	22.5	19.6	14.6	11.2	30.2	29.5
1.5	0.5	1.5	6.0	4.1	10.2	8.6	10.4	6.9	15.8	12.0
2.5	0.0	0.0	8.9	7.8	14.3	12.6	12.0	11.5	24.0	22.8
2.5	1.0	0.0	10.5	9.6	16.2	13.4	8.8	8.0	18.5	15.1
2.5	1.0	2.5	4.8	2.3	6.9	3.6	5.9	3.9	10.0	6.8
3.5	0.0	0.0	14.3	13.2	18.3	10.8	14.3	13.4	25.8	20.5
4.5	0.0	0.0	15.2	14.5	20.5	12.2	14.8	13.0	32.5	25.8
0.0	0.0	3.5	2.5	0.9	15.6	10.1	1.6	0.7	5.5	1.0
0.0	0.0	4.5	3.2	1.3	18.2	11.5	1.0	0.5	6.8	1.6

CIF(%)- callus induction frequency, ECR(%)- embryogenic callusing response

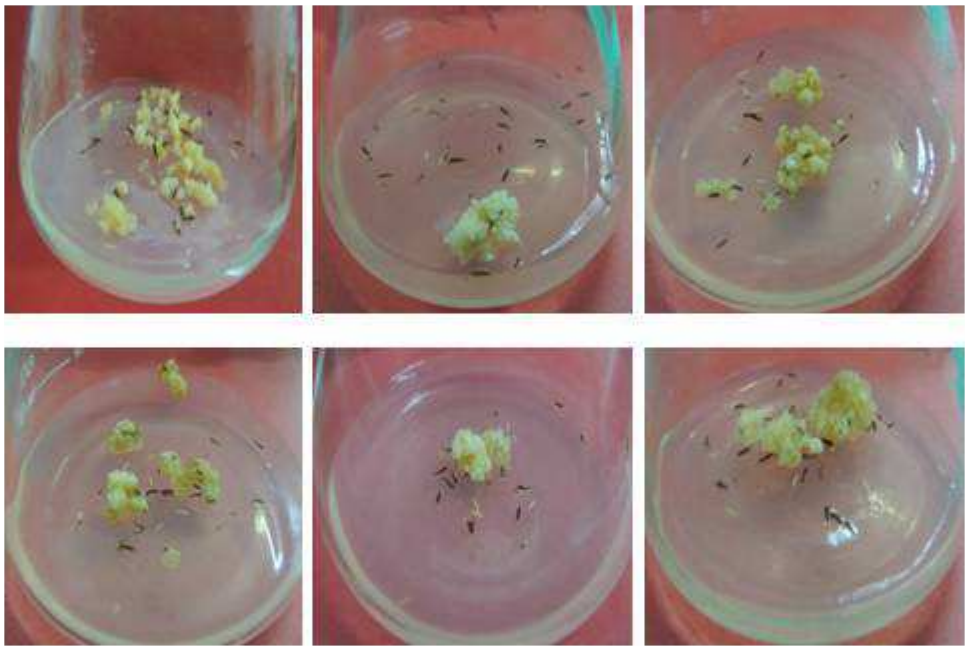


Fig. 2(a). Callus induction from anthers of F_1 of a cross Khandagiri x Dullar (Primary culture)

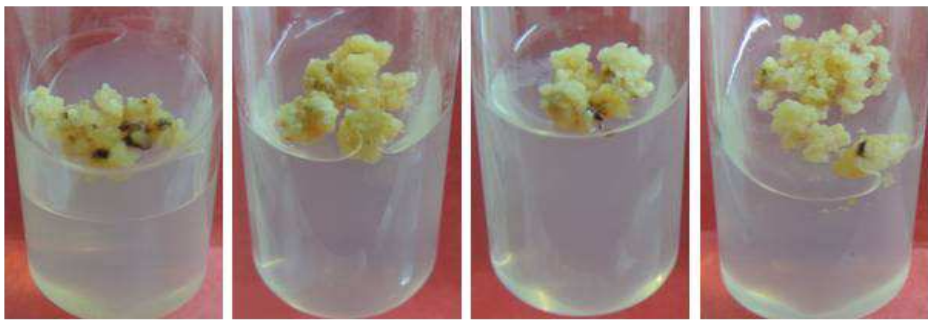


Fig. 2(b). Subculture of anther derived callus of a cross Khandagiri x Dullar

4.2 Anther culture response for callus induction

Haploid plant production was first reported in rice through anther culture by Niizeki and Oono (Niizeki and Oono, 1968). Limited morphogenetic potential of anther derived calli and a higher percent of regenerated albino plants (Roy *et al*., 2005, Talebi *et al*., 2007) seem to be major constraints for double haploid breeding.

N6, MS and SK1 solid media are *in vogue* used for anther culture and the former being 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 embryogenic callusing from rice anthers (Trejo-Tapia *et al*., 2002; Herath *et al*., 2008; Lal *et al*., 2014; Mukherjee *et al*., 2015). In the present investigation, anther culture response was assessed using N6, SK3, MS and CIM medium supplemented with

different concentration of 2,4-D, Kn and NAA (Table 2, Fig 2a, 2b). N6 was originally formulated for anther anther culture. In general, SK3 (a modification of N6) recoded higher callus induction frequency than N6 in the present cross combination of indica rice. However, CIM (a formulated modification of MS) had revealed improved callusing response. 1.5mg/l 2,4-D + 0.5mg/l Kn induced better callusing response across all the media used and CIM revealed appreciably high callus induction frequency (30.2%) and highest embryogenic callusing response (29.5%) among the media. In this context, 0.5mg/l 2,4-D + 2mg/l Kn + 3.5mg/l NAA followed by 4.5mg/l 2,4-D in CIM medium also revealed high callus induction frequency and embryogenic callusing response. Somatic embryogenic callus are in vogue expected to induce higher regeneration response. Hence, CIM supplemented with 0.5mg/l 2,4-D + 2mg/l NAA may be considered ideal for callusing response in anther culture of indica rice. The F_1 hybrids are more responsive to anther culture than their parents. Herath *et al*. (2007) recorded highest callus induction frequency (29.4%) in N6 medium for F1 hybrid Hu Lo Tao × BG 90-2. Dash et al. (2014) reported a callus induction frequency of as high as 37.83 % from anther culture of a cross CRMS31B x CRMS24B. Nirouli and Bimb (Nirouli and Bimb, 2009) reported higher callus induction frequency in N6 medium with 2.5mg/l 2,4-D(2,4-dichloro-phenoxyacetic acid) + 0.5mg/L Kinetin(Kn) than N6 + 4mg/L NAA(Napthalene acetic acid) + 0.5mg/L Kn.

Table 4. Embryogenic plantlet regeneration response from anther culture of a F_1 cross Khandagiri x Dular in RM media

Hormone recipe			Days first plantlets observed in RM	Embryogenic regeneration response (%)*			Freq. 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	3.8	0.0	1.57	No rooting
0.5	1.0	0.5	33	0.2	6.2	6.4	1.01	2.3	-do-
0.25	0.5	0.25	31	0.0	2.6	2.6	0.0	3.1	Rooting
0.25	0.75	0.25	28	0.4	17.8	18.2	1.85	12.1	-do-
0.5	2.0	1.0	38	1.3	2.5	3.8	1.05	3.0	No rooting
1.0	2.0	0.5	32	0.5	6.3	6.8	1.08	2.5	Less Rooting
2.0	0.0	0.5	30	2.8	5.2	8.0	3.58	6.0	-do-

0.0	2.0	0.5	40	12.8	0.0	12.8	6.1	0.0	Rooting
0.0	2.0	0.0	No response	0.0	0.0	0.0	0.0	0.0	-
2.0	0.0	0.0	No response	0.0	0.0	0.0	0.0	0.0	-
0.75	0.75	0.5	30	1.5	13.5	15.0	1.23	10.1	Rooting
1.0	1.0	0.5	35	0.6	5.2	5.8	0.0	1.5	-do-
1.5	0.5	0.0	No response	0.0	0.0	0.0	0.0	0.0	-
0.5	1.5	0.0	No response	0.0	0.0	0.0	0.0	0.0	-

*% of anthers respond to embryo formation in CIM+ 1.5mg/l 2,4-D + 0.5mg/l Kn and also follow- up embryogenic plantlet regeneration in RM medium.

Fig. 3(a). Organogenic regeneration of anther culture derived albino plantlets

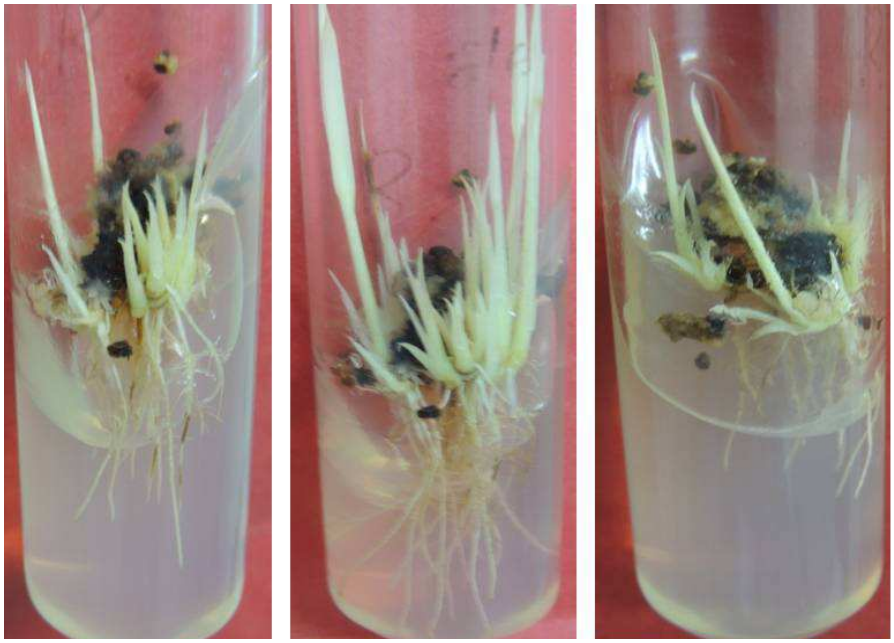


Fig. 3(b). Somatic embryogenic regeneration of anther culture derived albino plantlets

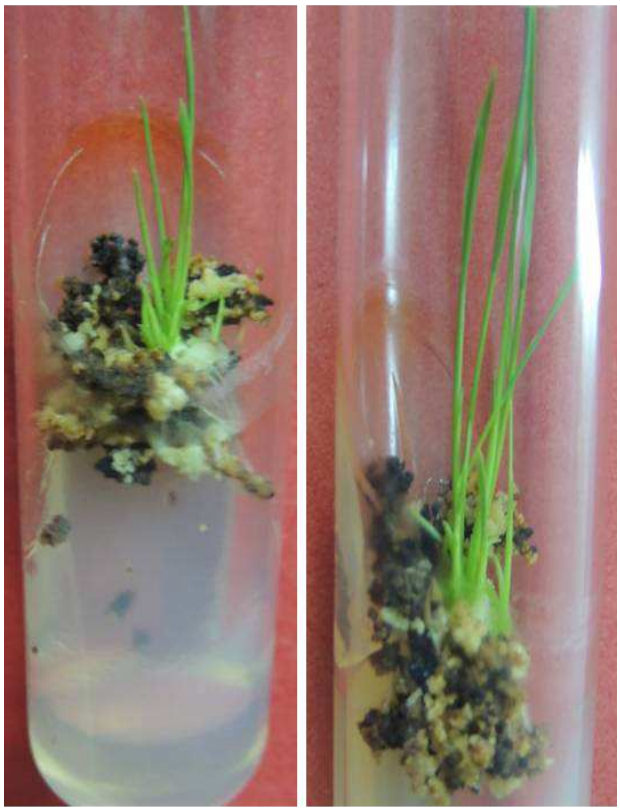


Fig. 3(c). Somatic embryogenic regeneration of anther culture derived green plantlets

Fig. 3(d). Plant establishment of anther culture derived green plantlet in pot mixture

4.3 Anther culture response for plantlet regeneration

Indica x indica hybrids produce more of albino plantlets compared to Indica x Japonica hybrids (Rangaswamy *et al*., 1992). Calli with higher levels of the peroxidase enzyme has been reported to display a greater regeneration potential than those with lower levels of the enzyme producing predominantly albinos (Subhadra *et al*., 1998). Induction of sporophytic haploidy in rice anther culture is in vogue controlled by haploid (gametophytic) inhibitor gene 'hap' (Kasha *et al*., 1983) 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*., 2014b).

Embryogenic calli developed from CIM with 0.5mg/l 2,4-D + 2mg/l NAA were transferred to the regeneration medium RM (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 elicited any plant

regeneration response (Table 3). However, inclusion of NAA with BAP or combination of BAP and Kn revealed plantlet regeneration (Fig 3a, 3b, 3c). Among different combination of hormone recipes, 0.25mg/l Kn + 0.75mg/l BAP + 0.25mg/l NAA induced highest frequency of plantlet regeneration in as early as 28 days of inoculation of anthers in primary culture, but almost all such plantlets were albinos. Besides, most of the treatments also induced higher frequency of albino plantlets with or without rooting. This seems to be a major setback in the prospect of anther culture in genetic improvement of rice. However, it is interesting to note that highest frequency of green plantlet regeneration (12.8%) with rooting was achieved in RM + 2mg/l BAP + 0.5mg/l NAA without any albino plantlet formation. The above media also resulted highest number (6 nos.) of green plantlets per responsive callus. Besides, 2mg/l Kn + 0.5mg/l NAA in RM medium also induced green plantlet regeneration though in low frequency (2.8%). Rout *et al.*, (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 (0.5mg/1), BAP (2 mg/1) and NAA (1 mg/1) at ratio of 1:4:2. Xa and Lang (Xa *et al*., 2011) reported 6.17% to 14% regeneration from four crosses in MS medium with combination 1mg/L BA(6 benzyl aminopurine) + 2mg/L Kinetin + 3% sucrose.

Fifty-five plants (44.7%) out of 123 anther culture derived plantlets from the rice hybrid Khandagiri x Dular had come to flowering and normal maturity. These anther culture derived plants maintained normal growth without any abnormality and set seeds upon flowering. Rest of the plantlets either did not survive during hardening and plant establishment or showed abnormal growth without flowering (Fig 4). The DH lines resulted from these selected DHs exhibited 100% uniformity and stability in morpho-agronomic traits which confirmed their DH ploidy status. Allah *et al*. (2014) obtained 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.

Fig. 4. Anther culture derived abnormal plants

4.4 Identification of elite drought tolerant DH lines

The complex nature of drought tolerance, genotype × environment interaction, and the difficulty of effective drought tolerance screening complicate the development of drought tolerant genotypes. Several varieties that have been widely grown in rain-fed environments in India for many years are indeed lack of drought tolerance leading to low productivity. The cultivation of upland rice is a non-flooded alternative to lowland rice culture that can reduce the demand for irrigation water by 50–70% system, tall stature, thicker stem and fewer tillers. These characteristics are believed to confer adaptability to the upland conditions. It is reported that the long and thick root system of upland rice contributes greatly to its drought resistance. Additional studies have shown that the ratio of root weight to shoot weight, and root penetration ability is also correlated with drought resistance. Identification of useful markers linked to drought resistant trait(s) would help to develop drought tolerant rice genotypes using marker assisted selection.

Farmers used to cultivate early rice varieties in the upland situation which are sown at onset of monsoon and harvested latest before 15th October. Cultivation of these varieties entirely depends upon seasonal rainfall during Kharif. Therefore, genotypes chosen for upland situation need to have drought tolerance to combat frequent drought spell during kharif season. In recent years, vagaries of monsoon warrant selection of rice varieties for suitability of cultivation with minimum water availability. Therefore, in the present pursuit, an attempt was taken to screen the available 55 double haploid lines derived from a cross Khandagiri x Dular.

Table 5. Assessment of drought tolerance at seedling, vegetative and flowering stage

Genotype	Seedling stage[a]			Vegetative Stage[b]			PI stage[b]
	Leaf drying score	Root length	Shoot length	Leaf Rolling Score	Leaf drying score	Drought recovery score	Leaf drying Score
DHL1	2.7	8.5	6.5	1.8	1.6	2.6	1.2
DHL2	5.7	4.5	6.0	2.8	2.6	7.0	8.0
DHL3	6.7	4.5	5.5	4.8	1.6	8.2	7.2
DHL4	2.0	10.0	9.5	1.8	2.6	0.3	1.5
DHL5	8.7	6.5	4.5	1.8	1.2	9.0	3.0
DHL6	7.0	8.0	8.5	8.8	5.6	7.0	3.2
DHL7	3.7	6.5	7.5	4.8	4.6	5.3	8.0
DHL8	3.0	8.0	10.0	1.3	1.8	1.2	1.0
DHL9	4.0	5.8	7.2	2.8	2.6	3.0	5.0
DHL10	3.3	3.5	6.5	2.8	1.6	5.2	9.0
DHL11	7.0	5.5	5.5	4.8	6.6	5.0	9.0
DHL12	0.5	12.2	9.5	0.8	2.8	3.2	5.0
DHL13	2.0	7.5	6.5	2.5	2.8	3.0	9.0
DHL14	4.7	9.0	9.0	2.8	2.2	4.0	9.0
DHL15	9.0	9.5	8.5	9.3	7.8	9.0	3.0
DHL16	9.0	9.0	6.0	2.3	1.8	9.0	9.0
DHL17	4.3	7.0	7.0	1.3	1.4	3.0	6.0
DHL18	2.7	5.0	7.0	5.3	4.8	5.2	5.2
DHL19	5.7	8.5	10.0	7.3	3.8	9..0	9.0
DHL20	8.7	8.5	7.5	4.8	4.6	9.0	9.0
DHL21	5.3	7.0	7.0	2.3	2.8	8.2	1.0
DHL22	3.7	8.5	8.0	1.3	1.8	5.0	9.0
DHL23	5.3	2.5	8.0	3.3	2.8	8.2	7.0
DHL24	2.0	10.2	10.5	0.8	1.6	0.4	1.0
DHL25	7.7	10.0	11.0	5.3	4.8	9.0	9.0
DHL26	7.7	5.4	9.5	5.3	5.6	8.5	8.2
DHL27	8.7	6.0	5.5	1.3	2.6	8.8	7.5
DHL28	4.0	6.0	9.5	0.8	1.6	5.6	5.2
DHL29	6.3	7.0	8.0	3.3	7.8	9.0	2.4
DHL30	1.2	12.5	15.0	1.3	1.6	2.2	1.0
DHL31	4.0	8.0	6.0	1.3	1.6	6.2	6.3
DHL32	3.3	8.5	8.0	0.8	1.6	5.6	3.2
DHL33	6.0	4.5	8.5	1.3	2.6	6.2	3.4
DHL34	6.0	6.0	7.5	3.3	2.6	6.0	4.2
DHL35	7.3	3.5	5.0	1.1	2.6	7.0	9.0
DHL36	6.7	6.0	5.5	5.3	4.6	8.0	8.0
DHL37	3.3	8.5	8.5	5.3	5.6	5.2	8.2
DHL38	6.0	8.0	7.0	6.3	6.6	9.0	9.0
DHL39	5.3	9.5	8.5	6.5	4.8	8.6	6.3
DHL40	4.0	9.0	9.0	2.5	2.5	8.4	4.2

Genotype	Seedling stage[a]			Vegetative Stage[b]			PI stage[b]
	Leaf drying score	Root length	Shoot length	Leaf Rolling Score	Leaf drying score	Drought recovery score	Leaf drying Score
DHL41	1.7	10.5	9.0	1.3	2.6	0.5	1.2
DHL42	2.0	8.0	7.0	8.0	7.5	2.0	9.0
DHL43	1.0	12.0	9.0	1.3	1.6	1.0	0.8
DHL44	3.0	12.5	10.0	2.5	4.8	3.2	9.0
DHL45	3.3	4.0	9.0	3.5	4.8	5.0	9.0
DHL46	4.7	9.5	9.0	3.5	1.8	7.2	9.0
DHL47	6.7	8.0	9.0	3.5	4.8	7.0	9.0
DHL48	2.0	10.0	11.5	0.5	2.8	0.5	1.0
DHL49	4.3	9.5	6.0	2.5	1.8	7.2	9.0
DHL50	2.7	9.5	9.0	6.5	2.8	3.2	2.2
DHL51	2.7	10.0	9.5	2.5	1.8	5.6	9.0
DHL52	2.3	6.5	10.5	6.5	5.8	4.0	9.0
DHL53	0.7	12.5	9.0	0.5	0.8	0.4	2.3
DHL54	4.7	6.5	7.0	5.8	4.6	9.0	3.2
DHL55	3.7	8.5	9.0	4.8	5.6	6.3	6.5
Dular	0.2	12.8	10.0	0.5	0.8	0.4	0.5
Khandagiri	8.0	5.5	6..0	5.5	4.8	8.2	9.0
Mean	4.52	7.91	8.16	3.37	3.35	5.46	5.82
Range	0.2-9.0	2.5-12.8	4.5-15.0	0.5-9.3	0.8-7.8	0.3-9.0	0.5-9.0

a- Test of drought tolerance using PEG, b- Test of drought tolerance in pot culture in net house

Fig. 5. Selection of double haploids under artificial drought stress (using 30M PEG_{6000})

4.4.1 Artificial screening for drought tolerance using PEG

Drought tolerance was assessed on 0-9 scale, '0' being no symptoms of drought stress, while double haploid line(DHL) with score '9' was recorded most susceptible based on different parameters affecting growth of plants e.g., leaf drying, leaf rolling and drought recovery (after re-watering following drought stress).

Besides, seedling growth relating to shoot and root length under drought stress were assessed as compared to both the parents (Dular and Khandagiri). Data for each parameter were recorded on each successive day following drought stress and averaged.

Polyethylene glycol with molecular mass of 6000 and above are non-ionic,non-toxic, water soluble polymers which are not expected to penetrate intact plant tissues rapidly. It forms hydrogen bonds with water and thereby inhibits both water and mineral uptake by root (Sidari *et al*. 2008). Thus, such a osmotic agent acts in lowering the water potential in a way similar to soil drying (Kaydan *et al*; 2008). Thus, it is reasonable that PEG is capable to induce water stress at seedling stage which can be correlated with that in the field or the green house

.At seedling stage, drought stress was imposed to seedlings (grown in plastic cups) using PEG_{6000} and the DHLs were scored based on leaf drying score and seedling growth relating to root and shoot(Fig 5). Leaf drying from tip of the upper leaves seems to be the immediate symptom under drought stress. It ranged from 0.2 to 9.0 with the least score recorded in cv. Dular (drought resistant parent) and the maximum score (9.0) in the DHL15 and DHL16 (Table 4). Inherent drought tolerance was shown to be associated with DHL 12, DHL 43 and DHL 53 with leaf drying score (LDS) $\leq$ 1.0.

Higher root length is in vogue associated with drought tolerance in upland rice. In the present investigation, DHL 12, DHL 30, DHL 43, DHL 44 and DHL 53 recorded root growth $\geq$ 12cm and were comparable to the drought resistant parent 'Dular' (12.8cm.). Shoot length ranged from 4.5cm. in DHL 5 to as high as 15.0cm. in DHL 30. Under drought stress, a part of the energy (ATP) which otherwise could have been used for vegetative growth, is diverted to combat against the moisture stress. Therefore, it is reasonable that growth of seedlings and also seed yield become reduced even in most drought resistant genotypes under moisture stress compared to their status in normal condition. In this context, DHL 24, DHL 25, DHL 30, DHL 44 and DHL 48 maintained simultaneously higher root and shoot growth and these were comparable to Dular (12.8cm. and 10.0cm.) under similar moisture stress. However, Allah *et al.* (2014) studied a set of 40 DH lines and indicated that germination percentage could be considered as one of the beneficial drought tolerance indicators.

Fig. 6(a). Pot culture of double haploids for drought tolerance screening

Fig. 6(b). Selection of double haploids based on Leaf drying at vegetative stage.

Fig. 6(c). Selection of double haploids based on drought recovery score at vegetative stage

Fig. 6(d). Selection of double haploids based on Leaf drying at PI stage

4.4.2 Screening for drought tolerance in net house (pot culture)

It is difficult to evaluate drought tolerance in the field as the difficulty stems from the inherent spatial variability of soil moisture stress, the "complex" inheritance of drought tolerance traits. Therefore, a set of 55 DH lines were tested in pots lined with polythene to provide homogeneous moisture stress conditions.

Drought stress tolerance among DHLs was scored on 0-9 scale (as recommended by IRRI) at vegetative and panicle initiation stage (in pot culture) under controlled moisture stress for three consecutive days followed by re-watering to assess drought recovery status. Leaf rolling is *in vogue* a natural contrivance to restrict transpiration loss. It is noted that some of the DH lines e.g., DHL 12, DHL 24, DHL 32, DHL 48 and DHL 53 remain with fully expanded leaf or exhibited temporary leaf rolling under intense sun light and become unfolded at night and morning. These DH lines recorded minimum leaf rolling score (Table 4) and were considered drought tolerant. In contrast, the drought sensitive parent cv. 'Khandagiri' and most of the DH lines showed permanent leaf rolling followed by whole plant leaf drying in the follow-up days under drought stress. When watering was rescheduled following drought stress; a few of the DH lines e.g., DHL 4, DHL 24, DHL 41 and DHL 48 which initially had shown a bit drought symptoms, but again recovered their normal growth to a greater extent. Extent of leaf drying was assessed at both vegetative and PI stage; and some of the DHLs with minimum drying of leaves were DHL 1, DHL 24 , DHL 30 and DHL 43. Blum *et al*.(1999) imposed two consecutive drying cycles between tillering and heading to 100 doubled-haploid lines of a cross CT9993-5-10-1-M/IR62266- 42-6-2 followed by full irrigation until harvest.. The DH lines differed significantly for all measured plant phenology traits. Besides, the plant stress indicators were significantly correlated across lines.

Some of the DH lines screened were noted to be different depending upon the parameter used and growth stage for drought tolerance. DHL 12 was found to be tolerant based on its tolerance status at seedling and vegetative stage, but such DH line turns to be drought sensitive at PI stage. Similarly, DHL 31 and DHL 32 which were tolerant at vegetative stage but found to be sensitive at both seedling and PI stage. The differential response of rice DH lines might be due to different mechanisms they harbour to combat the limited water stress condition. For instance, the DH genotypes which scored tolerant based on leaf rolling score, might have a strong

mechanism to restrict transpiration loss of water plausibly by equipped functioning of guard cells. Similarly, genotypes responded well for drought recovery might have efficient root characteristics while, those scored tolerant for leaf drying might have efficient mechanism for stability for synthesis of chlorophyll in the green foliage. However, considering different phenotyping criteria for drought tolerance e.g., extent of root growth, leaf drying, leaf rolling and drought recovery (after re-watering); DHL 1, DHL 4, DHL 8, DHL 24, DHL 30, DHL 41, DHL 43, DHL 48 and DHL 53 were considered drought resistant double haploid lines.

Table 6. Mean performance of double haploid lines for different morpho-economic traits.

Genotype	Days to 50% flowering	Days to maturity	Plant height (cm.)	No. of effective bearing tillers	Panicle length (cm.)	Grains/ panicle	1000-Grain weight (gm.)	Fertility Percent-tage	Seed Yield/ (q/ha)
DHL1	60.9*	90.8*	92.8*	320.1	22.4	90.6	20.8	83.0*	**42.2***
DHL2	63.9	92.8*	125.8*	305.1	21.2	31.2	15.5	20.3	19.7
DHL3	63.9	94.8*	126.8*	288.1	24.4*	65.5	16.7	60.0	20.5
DHL4	75.9*	106.8*	120.8*	225.1	24.5*	110.6*	21.2	85.0*	**45.2***
DHL5	70.9	102.8	130.8*	402.1	20.2	49.8	16.7	80.4	29.6
DHL6	70.9	100.8	115.8	189.1	23.9*	50.9	18.6	63.6	17.7
DHL7	60.9*	90.8*	100.8	203.1	20.4	79.6	11.6	68.8	18.2
DHL8	57.9	87.2*	109.1	326.5*	24.7*	103.3*	25.2*	80.4	**43.8***
DHL9	75.9*	105.0	134.8*	449.2*	19.4	36.9	16.8	85.6*	30.8
DHL10	68.9	95.8	100.8	395.5*	21.4	38.6	15.6	57.9	21.6
DHL11	65.9	96.0	104.8	240.1	21.6	70.8	13.8	76.4	22.4
DHL12	77.9*	106.0*	127.1*	318.4	23.5	82.3	17.8	82.4*	40.2
DHL13	73.9	105.3	126.1*	330.4*	22.3	52.8	20.9	90.0*	34.3
DHL14	68.9	97.3	123.8*	504.3*	19.3	37.8	15.1	88.7*	30.3
DHL15	67.9	96.8	128.1*	339.4*	21.3	50.3	16.7	56.8	28.0
DHL16	74.9	105.3	123.1*	217.4	23.8*	47.3	20.2	68.6	17.8
DHL17	63.9	94.3*	101.1	299.2	22.8	127.3*	14.8	89.2*	41.5
DHL18	65.9	95.8	89.1*	199.5	21.5	51.3	16.7	71.0	17.0
DHL19	77.9*	105.3	110.1	240.4	25.3*	59.3	23.9*	58.2	32.7
DHL20	61.9*	91.8*	122.8*	268.1	19.8	39.6	20.0	58.0	19.8
DHL21	101.9*	130.3*	110.1	328.2*	22.8	53.8	20.3	82.8*	34.6
DHL22	69.9	99.3	95.1*	426.4*	20.3	38.8	17.7	85.0*	32.3
DHL23	75.9*	104.3	115.1	293.2	22.7	55.8	21.5	78.1	32.6
DHL24	58.1*	88.3*	120.3*	313.4	25.0*	60.1	26.6*	80.6	**45.0***
DHL25	66.9	96.3	132.1*	438.2*	16.8	39.3	15.8	34.8	26.9
DHL26	76.1*	105.3	136.3*	270.4	23.8*	71.8	20.9	63.2	39.4
DHL27	58.1	88.3*	107.3	306.4	23.0	70.6	27.5*	85.8*	34.0
DHL28	80.1*	108.4*	109.3	362.4*	22.9	75.1	19.7	88.7*	40.2
DHL29	48.9	78.0*	96.1*	278.4	18.3	49.9	18.2	83.6*	25.0
DHL30	67.1	96.3	133.8*	325.4*	26.6*	120.1*	19.5	86.4*	**42.2**

Genotype	Days to 50% flowering	Days to maturity	Plant height (cm.)	No. of effective bearing tillers	Panicle length (cm.)	Grains/ panicle	1000-Grain weight (gm.)	Fertility Percent-tage	Seed Yield/ (q/ha)
DHL31	69.1	98.9	102.3	309.4	25.8*	64.8	20.6	81.6*	40.1
DHL32	68.1	99.6	120.3*	300.4	28.0*	82.1	22.9	81.3*	40.0
DHL33	73.1	102.6	118.5*	335.4*	23.6	36.8	22.4	37.6	29.6
DHL34	80.1*	110.3*	101.3	282.4	22.0	39.3	14.9	31.3	15.8
DHL35	85.1*	115.3*	111.6	341.4*	19.5	46.1	17.0	68.2	25.5
DHL36	64.1	93.3*	110.3	198.4	23.6	78.1	17.9	66.4	25.9
DHL37	79.1*	108.3*	102.3	381.4*	19.5	34.1	21.8	40.8	26.7
DHL38	57.1*	86.3*	115.3	316.4	20.6	50.6	26.4*	82.3*	38.5
DHL39	63.9	92.3*	122.6*	319.0	25.7*	115.3*	19.0	90.4*	33.3
DHL40	65.2	98.8	106.5	330.5*	20.4	50.5	15.4	85.6*	26.8
DHL41	57.1*	88.3*	101.9	307.4	23.3	103.1*	28.9*	94.2*	**46.6***
DHL42	64.5	98.5	86.2*	402.7*	21.5	69.7	21.9	58.2	37.9
DHL43	67.1	99.3	131.8*	381.4*	25.0*	90.1	21.5	93.8*	**45.1***
DHL44	73.9	105.3	101.6*	305.9	22.4	57.8	18.6	57.8	30.5
DHL45	74.9	104.3	92.6*	367.9*	20.5	31.6	17.2	35.4	26.3
DHL46	63.9	95.3	104.6*	390.9*	23.4	45.8	23.7*	75.7	34.6
DHL47	86.9*	117.3*	99.6*	343.9*	24.2*	60.2	19.8	66.0	32.0
DHL48	67.9	99.8	117.6	307.9	23.5	98.9	24.8*	84.5*	**44.0***
DHL49	70.9	100.3	122.6*	280.9	19.9	63.6	26.6*	72.4	33.8
DHL50	66.9	98.8	102.8*	244.9	24.2*	91.8	18.6	52.0	28.4
DHL51	73.9	105.3	100.8*	296.9	17.6	67.4	19.9	82.6*	35.0
DHL52	58.9	91.3*	125.6*	189.9	23.5	81.8	26.2*	86.0*	29.3
DHL53	68.1	97.3	122.3*	345.4*	28.3*	98.1	26.6*	91.0*	**45.6***
DHL54	61.9*	91.0*	100.8*	182.1	27.8*	40.6	29.0*	70.5	26.6
DHL55	59.9*	92.2*	110.8	229.8	23.7*	49.6	25.5*	71.5	29.2
Dular	70.9	99.2	122.1*	219.4	24.9*	70.3	25.7*	90.0*	30.7
Khandagiri	56.9*	88.8*	92..6*	325.9*	21.9	75.8	20.2	62.2	24.5
Mandakini	77.0*	106.0*	115.2	284.5	23.5	95.7	25.9*	79.7	29.4
Sahbhagi-dhan	69.0	100.4	107.5	304.0	22.4	92.8	21.9	78.7	38.0
Mean	68.9	99.0	112.5	309..0	22.6	66.0	20.4	72.2	31.8
Range	48.9-101.9	78-130.3	86.2-136.3	182.1-504.3	16.8-28.3	31.2-127.3	11.6-29.0	20..3-94.2	15.8-46.6
$CD_{0.05}$	6.8	5.5	10.8	18.9	1.2	6.3	1.0	2.1	4.1

***- significant at $P_{0.05}$, Best check- Sahbhagidhan.**

4.5 Mean performance of DH lines for morpho-economic traits:

The mean performance of 55 DH lines, two parental genotypes and two standard checks (Mandakini and Sahbhagidhan) for different morpho-economic traits were presented in Table-5. The mean performance of genotypes clearly spelt out the abundant scope for selection of valuable genotypes. Wide array of range variation in almost all characters further strengthens the probable effectiveness of selection. There

was appreciable shift in mean performance both towards positive and negative direction over the standard checks (Table-3) for most of the characters. Dhaliwal *et al*., (1997) also reported wide variation in DH lines from F_1 hybrids due to recovery of rare gene combinations (following meiosis) in a population size (Dhaliwal *et al*., 1997) of 200 double haploid plants. Among the standard check varieties, Sahbhagidhan was the highest yielder (38.0q/ha) and hence could be considered as best standard check variety in terms of productivity. DH lines which transgress the upper range of the best standard check (Sahbhagidhan) though they may occur at very little frequency provide the necessary base for the desired direction of selection. In the present investigation, major emphasis was laid on identification of superior high yielding genotypes under drought stress. Many often direct selection based on *per se* grain yield led to missing of valuable breeding materials which otherwise have potential genotypic worth for some specific traits. Therefore, the promising genotypes with respect to specific traits have been sorted out for their use in future breeding programme.

Genotypes with maturity duration within 90-100 days are *in vogue* preferred for suitability in upland condition. In the present investigation, days to 50% flowering and days to maturity (physiological maturity) ranged from 48.9 to 101.9 days and 78.0 to 130.3days respectively (Table 5). Among the DH lines DHL29, DHL24,, DHL 27, DHL 38 and DHL 41 recorded significantly early flowering at around 60days and attained maturity even within 90 days. Among these, DHL 29 took just 78days to mature and it can be a valuable material for breeding of rice varieties for upland situation.

Varieties with plant height around 100-110cm. is usually suitable for drought stress upland condition. The popular check variety included under study e.g., Mandakini had moderate plant height (around 115.2cm.), whereas, Khandagiri, was significantly shorter in height (92.6cm.) as compared to the best standard check variety Sahbhagidhan (Table 5). In this pursuit, DHL 8, DHL17, DHL 28 and DHL 41 revealed intermediate plant stature.

Among the test genotypes, DHL42, DHL22, DHL29, HL 47and DHL45 had shown plant height within 100.0cm. In contrast, DHL26, DHL5, DHL9, DHL25, DHL30 and DHL43 exhibited tall stature (>130cm.) which may not be suitable for upland situation.

DHL14, DHL9, DHL25, DHL22, DHL5 and DHL42 revealed significantly high tillering ability (> 400/m^2) among the test entries as compared to Sahbhagidhan. Panicle features including length, weight, compactness and grains per panicle seem to be important factors for high productivity. DHL53 and DHL52 excelled in panicle length (≥28.0cm.) among the test DH lines under study. DHL17 and DHL30 had shown significantly higher number of grains per panicle (>120).

A range of 11.6 to 29.0g was recorded for 1000- grain weight. Grain dimension determines the test weight of a genotype. A few of the test DHLs e.g., DHL54, DHL41 and DHL27 in the present pursuit had bold grains and therefore, recorded significantly high 1000-grain weight (>27g).

Flowering to grain filling seems to be a critical stage for upland rice for recovery of high grain yield under drought stress. Therefore, genotype with high grain fertility status under drought stress is an important criterion for screening of genotypes for drought tolerance. Among the present set of double haploid lines, DHL41, DHL13, DHL39, DHL41, DHL43, DHL53 and DHL 17 recorded significantly high grain fertility percentage even more than 90% and each of them were comparable to the drought resistant parent 'Dular'(90.0%) and also excelled over the best standard check variety Sahbhagidhan.

Grain yield of a genotype is an artifact which resulted from a combination of yield contributing ancillary traits and it depends upon the interaction with ecological/environmental condition under which the genotype is grown. Genotypes with combination of desirable ancillary traits along with drought tolerance would certainly excel in productivity under drought stress. In the present investigation, the overall mean grain yield/ha revealed a spectacular wide array of variation ranging from 15.8 to 46.6q/ha among the test genotypes. Significant yield reduction was observed under drought stress in majority of the DH lines as compared the drought tolerant parent and standard checks used. However, few DH lines e.g., DHL41, DHL 53, DHL43, DHL4 and DHL24 performed well with significantly high grain yield (>45.0q/ha) than the best standard check variety Sahbhagidhan. It is worth to mention that these high yielding DH lines have been also rated to have high degree of drought resistance at seedling, vegetative and flowering stage. Lanceras *et al*. (2004) revealed a wide variation in a doubled haploid (DH) population derived from a cross between CT9993(an upland *japonica*) and IR62266-42-6-2 (IR62266, an *indica*) for potential yield, osmotic adjustment (OA), and root characters such as deep and thick rooting

system. Allah *et al*. (2014) studied a set of 40 DH lines and indicated that number of panicles/plant and grain yield could be considered for selection of drought tolerant genotypes.

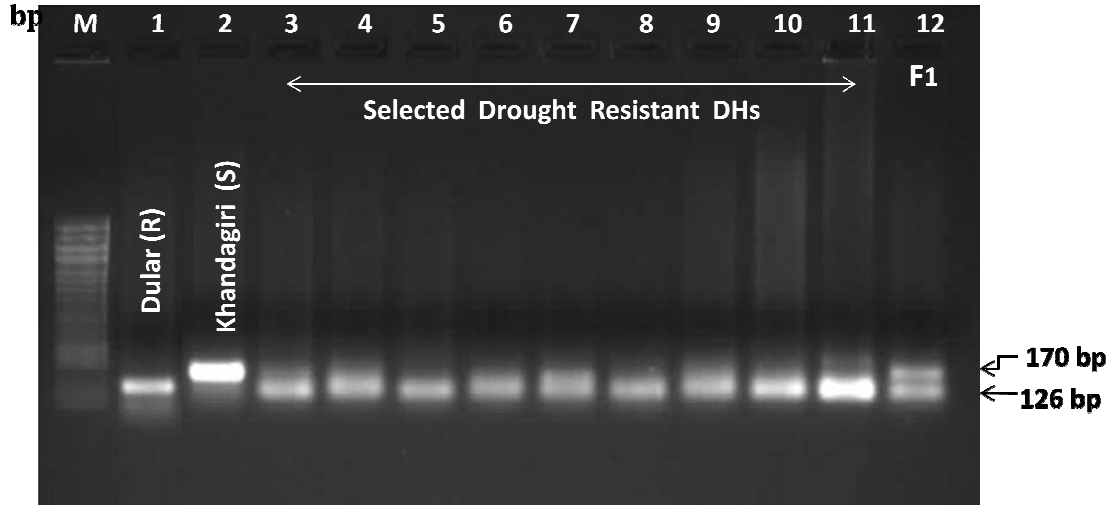


Fig. 7. Molecular profiling of parents, F_1 and selected drought resistant double haploids of a cross Khandagiri x Dullar using gene specific SSR primer RM 8085 (on chromosome 1). M: 100bp Mol. Wt. marker, Lane 1: Dular (drought resistant), Lane 2: Khandagiri(drought sensitive), Lane 3-11(drought resistant DH lines e.g., DHL 1, DHL 4, DHL 8, DHL 24, DHL 30, DHL 41, DHL 43, DHL 48 and DHL 53) and Lane 12: F_1 of the cross Khandagiri x Dular.

4.6 Marker aided selection of DH lines for drought tolerance

Many often, selection based on phenotyping for traits e.g., abiotic stress tolerance and quality traits (controlled by multiple genes or quantitative trait loci) with low heritability become misleading and unreliable. However, molecular markers could provide a useful tool to support phenotypic selection with utmost precision. A number of QTLs mapping studies covering whole genome are now available on drought tolerance that provided chromosome specific marker–trait associations using different mapping populations. Lang *et al* (2013) identified two QTLs for root length and another two QTLs for dry root weight using 229 lines (BC_2F_2), derived from a cross of OM1490/WAB880-1-38-18-20-P1-HB. Tripathy *et al*. (2000) detected nine QTLs relating to cellular membrane stability and Zhang *et al.* 92001) identified five QTLs relating to osmotic adjustment under drought stress using DH populations derived from the same cross CT9993×IR62266. Vikram *et al* (2011) identified a

major QTL $q_{DTY1.1}$ for yield on chromosome 1 using Bulk Segregant Analysis (BSA) of crosses N22/Swarna, N22/IR 64, N22/MTU 10.

DNA markers can be used to detect the presence of allelic variation in the genes underlying these traits. Ideally, the SSR markers are most preferred for molecular profiling and these should be highly polymorphic to discriminate genotypic variation in the breeding population. In recent years, marker aided selection has been attempted for drought tolerance breeding in a number of crop plants including rice.

In the present investigation, an attempt was taken to produce homozygous breeding lines (double haploids) with inherent *inter se* genetic variation for drought tolerance. Compared to F_2 and backcross populations, use of DHs permitted precise comparisons for drought tolerance with reduced confounding by genetic background. Besides, the unique complete homozygous nature of DHs, less time requirement to produce a large number of DHs, absence of heterozygosity, efficiency over conventional systems and absence of gametoclonal variation in DHs make them very valuable material for drought tolerance breeding. The paradox is that if few of these rice DH lines share a favourable trait, like drought tolerance, high yield, or even desirable quality features, they are likely to share the same DNA variation responsible for that trait.

Tolerance to drought stress is highly complex in nature. A number of primary traits (deep and thick roots, cell membrane stability and osmotic adjustment), secondary traits (relative water content, leaf water potential, canopy temperature, leaf death and rolling, panicle exsertion), integrative traits (harvest index, spikelet sterility, grain number per panicle, 1000-Grain weight, biomass, drought response index, flowering delay), phenology trait (flowering) and plant type traits (plant height and grain yield) cumulatively contribute to drought resistance in plants. Each of these traits is controlled by a set of QTLs. QTls for drought tolerance traits are located in chromosome 1, 4, 8, and 9. Most of the major QTLs (termed as meta QTLs) e.g., plant height, leaf drying, leaf rolling, root traits number, length, volume), panicle length, seed yield, grain weight, harvest index, stomatal conductance etc are reported to be colocated at a specific region (135.8-143.7cM) in chromosome 1 as per fine linkage map of McCouch *et al*. (2002) and Arvindkumar *et al* (2011). The said chromosomal site is marked by SSR marker RM 212, RM 302, RM 8085 and RM 3825. Among these, RM 8085 is located at close viscinity/ within almost all above

QTLs. Therefore, RM 8085 was considered for genotyping the erstwhile mentioned selected elite drought resistant DH lines (DHL 1, DHL 4, DHL 8, DHL 24, DHL 30, DHL 41, DHL 43, DHL 48 and DHL 53) along with their parents and F_1 for drought tolerance. The parent variety 'Dular' revealed the characteristic 126bp drought resistant allele, while Khandagiri had shown about 170bp amplified product and F_1 produced both the above alleles. It was interesting to note that all above selected DH lines had shown the drought resistant allele which confirms their drought resistance status.

In general, drought tolerance is *in vogue* associated with traditional tall indica genotypes. Dular and N22 having the drought tolerance allele of qDTY1.1 (Vikram *et al*. 2011), also possess the tallness allele of the sd1 gene (semi-dwarf 1 gene) for gibberellic acid synthesis (OsGA20 oxidase) (Vikram *et al*. 2016). Mandakini –a moderately tall genotype has appreciable drought tolerance among all improved upland rice genotypes bred in OUAT. In the present investigation, nine DH lines detected as drought resistant using chromosome 1 specific SSR marker RM 8085, had intermediate to tall plant height with high yield performance under drought stress (Table 5) and therefore, these DH lines might have the major QTL locus qDTY1.1 in Chromosome 1. The qDTY1.1 effect on grain yield under drought might be either because of the pleiotropic effects of the sd1 gene itself or due to a separate locus within the qDTY1.1 region tightly linked to sd1. It is reasonable that the genomic alterations that occurred at the qDTY1.1 or sd1 locus and other qDTY loci (due to selection) have contributed to the increased drought susceptibility. This suggests that there is always a chance of loss of drought tolerance while breeding high yielding dwarf rice genotypes.

Considering important morpho-economic traits, the drought resistant DHL 41can be selected as most suitable genotype for upland situation as it had semi-dwarf stature (101.9cm), matured within 90 days and exhibited significantly higher number of grains/panicle with very high 1000-grain weight(28.9g), fertility percentage(94.2%) and seed yield(46.6q/ha). Such a valuable DH line may be sorted out for rapid multiplication and commercial cultivation as a substitute to the popular high yielding variety 'Khandagiri' and even the ruling drought tolerant variety 'Sahbhagidhan'. Further, the high yielding DH lines identified in this pursuit may be of emense value for future breeding programme for augmentation of productivity *per se* under drought stress.

SUMMARY AND CONCLUSION

India has large diversity of rice germplasm and in particular the Jaypore tract of Odisha is considered as secondary centre of origin. The availability of large number of land races pertaining to different ecological situation are still amenable for strategical research particularly for adverse climatic situation e.g., drought, cold, high temperature and soil nutritional stresses. Considering the prevailing paradigm shift in agro-climatic situations in different parts of india, plant scientists must be prepared to face the challenging task to provide food and nutrition for the ever-increasing world population and Asia in particular.

In this context, an attempt was taken to develop a set of double haploid lines from a cross Khandagiri (drought sensitive)x Dular(local land race with known drought tolerance) with a view to generate completely homozygous breeding lines amenable for identification of drought resistant genotype(s) and follow up multiplication within a period of two years.

A hormone recipe comprising 1.5mg/l 2,4-D + 0.5mg/l Kn induced better callusing response from anthers across all the media used and CIM revealed appreciably high callus induction frequency (30.2%) and highest embryogenic callusing response (29.5%) among the media. In this context, 0.5mg/l 2,4-D + 2mg/l Kn + 3.5mg/l NAA followed by 4.5mg/l 2,4-D in CIM medium also revealed high callus induction frequency and embryogenic callusing response.

Embryogenic calli developed from CIM with 0.5mg/l 2,4-D + 2mg/l NAA were transferred to the regeneration medium RM (a modification of MS) with different combination of Kn, BAP and NAA for plantlet regeneration. Among different combination of hormone recipes, 0.25mg/l Kn + 0.75mg/l BAP + 0.25mg/l NAA induced highest frequency of plantlet regeneration in as early as 28 days of inoculation of anthers in primary culture, but almost all such plantlets were albinos. However, highest frequency of green plantlet regeneration (12.8%) with rooting was achieved in RM + 2mg/l BAP + 0.5mg/l NAA without any albino plantlet formation.

Altogether 55 putative DHs out of total 123 anther derived plantlets came to normal flowering and produced filled grains. Such *in vitro* derived DH plants were harvested individually to raise DH lines to be used as a mapping population in future

studies. Besides, a part of seeds harvested from individual anther culture derived DH plants was used to assess their status for drought tolerance at seedling, vegetative and flowering stage; and also morpho-economic traits including seed yield under drought stress. The selected drought tolerant high yielding DH lines were further confirmed for their drought resistance status using molecular profiling based on the most closely linked molecular marker RM 8085 on chromosome 1.

Considering different phenotyping criteria for drought tolerance e.g., extent of root growth, leaf drying, leaf rolling and drought recovery (after re-watering); DHL 1, DHL 4, DHL 8, DHL 24, DHL 30, DHL 41, DHL 43, DHL 48 and DHL 53 were considered drought resistant double haploid lines.

Molecular profiling using SSR marker RM 8085 revealed the characteristic 126bp drought resistant allele in 'Dular', while the drought sensitive parent 'Khandagiri' had shown about 170bp amplified product and F_1 produced both the above alleles at RM 8085 locus. All above nine selected DH lines had shown the drought resistant allele which confirms their drought resistance status.

Among the present set of double haploid lines, DHL41, DHL13, DHL39, DHL41, DHL43, DHL53 and DHL 17 recorded significantly high grain fertility percentage even more than 90% and each of them were comparable to the drought resistant parent 'Dular'(90.0%) and also excelled over the best standard check variety Sahbhagidhan.

A few DH lines e.g., DHL41, DHL 53, DHL43, DHL4 and DHL24 performed well with significantly high grain yield (>45.0q/ha) than the best standard check variety Sahbhagidhan. These high yielding DH lines have been also rated to have high degree of drought resistance at seedling, vegetative and flowering stage and also rechecked for their drought resistance status using molecular marker.

Considering important morpho-economic traits, a drought resistant line DHL 41can be selected as most suitable genotype for upland situation as it had semi-dwarf stature, matured within 90 days and exhibited significantly higher number of grains/panicle with very high 1000-grain weight, fertility percentage and seed yield. Such a valuable DH line may be sorted out for rapid multiplication and commercial cultivation as a substitute to the popular high yielding variety 'Khandagiri' and even the ruling drought tolerant variety 'Sahbhagidhan'. Further, the high yielding DH lines identified in this pursuit may be of emense value for future breeding programme for augmentation of productivity *per se* under drought stress.

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