Review Paper

A review on anther culturing an effective tool in double haploid production

ABSTRACT

At present, anther culture is a ground-breaking technique for plant breeding. Haploids are plants with a gametophytic chromosome number and double haploids will be haploids that have experienced chromosome duplication. The development of haploids and double haploids (DHs) through gametic embryogenesis permits a solitary advance improvement of homozygous lines from heterozygous guardians, shortening the time required to create homozygous plants in correlation with the modern reproducing strategies that take lesser time than conventional methods. The anthers of plants are taken and treated with cold temperature. Further the explant is sterilized with disinfectants, dissected in laminar flow and placed on the media. At specific interval culture were transferred on the regeneration, shooting and rooting media, then chromosomes are counted for conformation. Chromosomes are doubled with colchicine treatment. There are a few accessible techniques to acquire haploids and double haploid production DHs, of which in vitro anther culture is the best and widely utilized. Final, double haploids play important role in product enhancement through genome mapping, quantitative locus investigation, hereditary modification/manipulation and focuses for genome change programs. This review article deals with the complete methodology of double haploid production by anther culture, its effectiveness over other techniques and its uses.

Keyword: Anther culture, haploids, double haploids, modern breeding, homozygosity.

INTRODUCTION

The varietal development is very tedious and time taking process through old conventional techniques because it includes the steps like screening, development of lines, evaluation, recognition and crossing for getting highly homozygous lines [1]. Infinite capitals of water land and fertilizers it is very difficult to cope the major challenges faced by the main food crops e.g. maize, wheat and rice in limited time. Enhanced production goals can be acquired through modern biotechnological tools and statistical techniques coupled with innovative breeding strategies [2]. The breeding techniques can be enhanced by the use of double haploid production

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delete techniques particularly in corn and barley [3]. DH production has been used in tobacco, barley, canola, wheat, rice and maize [4]. Double haploid production is referred as third crucial revolutionary methodology. Rewrite The former being hybrid seed technology and offseason nurseries [1]. The success rate of double haploids vary crop to crop and its success depends on the efficiency of the methods used for the production of the haploids.

The plants having half number of chromosomes in their cells are called haploid plants. Haploids occur naturally in the population but their probability to occur is very less. Naturally occurring haploid was first found in *Datura stramonium* L. [5]. In maize, first time haploidy was initiated by Chase in 1952 [6]. They can also be produced artificially through methods such as wide hybridization, chromosome elimination and pollination with mutated pollen (in vivo). This can also be induced through immature gametophyte culturing [7]. Haploids can be subjected to delete some agents such as colchicine and its chromosome number is doubled and it is called doubled haploid(2n=2x). Colchicine is a chemical that is widely used in transformation of haploids into doubled haploids through chromosomal doubling in vivo and some chemicals such as acenaphthene has also been used for the same purpose in tobacco [1]. The timing and quantity of application of these chemicals has direct consequences on the rate of regeneration and percentage of delete green plants/plantlets [8].

Development of plants from the male parts/ pollen grains (androgenesis) is indicating the totipotency of the plant and is very successful process. It is acclimatization that is present in the plants and only express in the climatic severities [7]. Embryogenesis from unfertilized gametes (female parts) such as ovaries or ovules (Gynogenesis) is not very popular as it is least efficient method but it can be used in the crops where other techniques are not working well [9]. The regeneration of viable plants through pollens has been found in 200 species belonging to different families [7] Please check reference. A lot of modern genetic techniques such as quantitative trait loci and gene association have gained advantage from the doubled haploids [10]. Anther culturing is preferred for doubled haploid production because it is an easy and simple method and it allows the experiment to be conducted at large scale and can be done in different crop species. While other techniques like isolated microspore culture and others require dexterous manpower and complex apparatus as compared to anther culturing [7]. Anther culturing for doubled haploids is effective because higher number of anthers are produced and

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occurrence of doubled haploids is more probable than other methods [11]. Plants when obtained from anther culturing followed by doubling of chromosome number have fixed genes (dominant or recessive) and offer ease of selection according to their suitability [12]. Through doubled haploid production not only homozygous lines can be obtained but the time for obtaining homozygosity is condensed to merely some/replace months [13]. The efficiency of anther culture for doubled haploid production is dependent on factors such as genotype used, growth conditions, pretreatment of spikes and developmental stages of pollens or anthers [14]. This review explains haploid and double haploid production through anther culturing and complete procedure for conducting experiments.

Anther culturing

Conventional recombination breeding begins with hybridization between parents take 6-9 cycles and 3-5 years of field evaluation before a breeding line and release as a new variety. In 1968, Niizeki and Oono were produced first haploids by anther culturing [15] in crop/plant name. Anther culturing is a suitable technique that fix homozygosity even in F_1 [16] and haploids improved by colchicine treatment. DHs line can bypass the inbreeding process [7] and has been successfully improved high yield and grain quality [17].

History of anther culture for haploid and double haploid production:

In 1921, First time Bergner observed sporophytic haploid in a weed species *Datura stramonium* L. [7]. Haploids importance was recognized in plant breeding and genetic research. Dunwell, 2010 reported the occasional haploids in range of species [18]. All angiosperm crops did not response embryogenesis induction but wheat (*Triticum aestivum L.),* tobacco (*Nicotiana spp*.), barley (*Hordeum vulgare L*.) and rapeseed (*Brassica napus L.)* are model species to study microspore embryogenesis because of high regeneration efficiency [9] and members of legume family or many woody plants, are remain recalcitrant to this type of in vitro morphogenesis [19] [20]. Researchers developed efficient techniques for haploid and DH production with an increasing number of genotypes and practical experiences of pollen embryogenesis protocols are used [9]. About 300 new varieties have been developed belonging to various families of the plant kingdom [7]. Mostly haploid derived varieties have been developed in following crops,

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asparagus, barley, Brassica, eggplant, melon, pepper, rapeseed, rice, swede, tobacco, triticale and wheat. [21] reviewed the intellectual property (IP) protection and the patenting system of haploid plants.

Anther collection stage:

Anther culturing efficiency can be improved by correctly identifying maturity stage of anther in indica rice varieties [22], wheat [23], barley [24] and bud of chickpea [25]. Development of anther stage different and depend on the genotypes of species. The development stage of pollen is commonly tested in one anther per floral by the acetic-carmine method. The anthers at different stages of development and prepared in acetocarmine staining solution (1% acetocarmine in 45% acetic acid) or DAPI (40, 6-diamidino-2-phenylindole dihydrochloride). To observed under microscope and to determine the stage of pollen development like in tobacco bicellular stage, in some crops late uninucleate to early-bicellular pollen grains and pollen at uninucleate stage produce mostly haploids plants [7]. Tetrad stage is not suitable for anthers culturing and after first pollen mitosis it falls fastly, starch deposition begins, sporophytic development stop and macroscopic structures do not form in microspores. At this stage, cold treatment can deviate from normal growth and development. Developmental stage of pollen is important to optimize the anther culture [26] [27]. [28] reported convenient morphological marker that is a distance from flag leaf to penultimate leaf auricle and estimated the maturity stage of pollen.

Preparation of explant:

The experiments can be conducted in screen house, net houses, green house and field [29]. Mostly researchers used screen houses [30], net houses [29] and field [31]. Booting stage (mid uninucleate at reduction division stages) were used for collecting the anthers according to method of [32]. [33] take panicles of rice with boot leaf sheath and washed with tap water, spread with 70% ethanol, cover with moist tissue paper, kept in polythene bag and cold shock at $8^{o}C$ for 8 days. On the day of culture, panicles were sterilized with freshly prepared 0.1% $HgCl_2$ solution for 10 minutes. Again, four times wash with distilled water. [30] cold-treated to panicles

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at 8-10°C for 7-10 days. After 10 days, sterilized surface with 20% (v/v) chlorox for 20 minutes. [34] took explants at booting stage and wrapped in moist tissue paper, cover with aluminum foil and kept in refrigerator 8-10 days (treatment uniform) for cold treatment to panicle. Antisepticise sample with 70% alcohol for 2 minutes and 0.1% $HgCl_2$ solution for 8 minutes and many times clean with distilled water. [31] did not wash explant and wrapped panicles in moistened water. Cold pretreatment at 12°C, 15 days in darkness. On culturing day, panicles sterilized with 94% ethanol and flame sterilized for a few seconds. In wheat, [23] took tillers with spikes and clipped off at surface level and tagged. They were kept in water for 6-8days (treatment uniform) at 4°C in dark environment. Spikes inside flag leaves were sterilized with 20% chlorax solution for 7 min and rinsed 3–4 times (treatment uniform) in sterile water. In barley, [24] cut spikes with flag leaf from inflorescences node and kept in beaker containing solution 5.0 mg L^{-1} $CuSo_4.5H_2o$ and 62gL^{-1} mannitol in the dark at 4°C for 4 days. After it remove flag leaf from the spikes and placed in sterilized petri dish and small amount (15mm) kept to rise the humidity. In chickpea, [25]took buds of different size and store in plastic container were kept in refrigerator at 4°C for 3-4 day. Then selected those buds at uninucleate stage and washed with buffer bleach for 20 minutes. Sterilization of anther and Anther culturing were done in laminar air flow.

Media for anther culturing:

Culture medium provides nutrition and also decides the microspores and having important aspect in androgenesis. Most common used media MS [35] and N6 [36] for anther culturing of cereals shown in table 1.1. Since 1978, several medium formulations are reported inducing N6-like Heh-2, Heh-5, SK-3, SK-8, Szechuan medium, Chaleffs R-2 and Medium V shown in table 1.1 [37]. In cereals, Low inorganic ammonium nitrogen (NH4+) had proved too good for androgenesis and N6 medium containing low NH4+ is using for rice anther culture. N6 media with organic ingredients like casein hydrolysate, yeast extracts and coconut water show enhanced androgenic callus induction [15] [33].

Table 1.1

Table 1.1: Media used for anther culturing.

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Components	N6 (mg/L)	B5 (mg/L)	He2 (mg/L)	SK1 (mg/L)	Components	MS mg/L
(NH4)2SO4	463	134	231	231	N2H4O3	1650.000
KNO3	3535	3125	3181	3180	KNO3	1900.000
KH2PO4	400	0	800	540	KH2PO4	170.000
NaH2PO4.H2O	0	150	0	0	-	-
MgSO4.7H2O	185	250	3.5	185	MgSO4	180.690
CaCl2.2H2O	166	150	166	440	-	-
H3BO3	1.6	3	1.6	6.2	H3BO3	6.200
MnSO4.4H2O	22.3	10	22.3	22.3	MnSO4.H2O	16.900
ZnSO4.7H2O	8.6	2	1.5	1.5	ZnSO4.7H2O	8.600
Na2MoO4.2H2O	0.25	0.25	0.25	0.25	MoO3·H2O	0.213
KI	0.8	0.8	0.8	0.8	KI	0.830
CuSO4.5H2O	0.025	0.025	0.025	0.025	CuSO4.5H2O	0.025
CoCl2.6H2O	0.025	0.025	0.025	0.025	CoCl2·6H2O	0.025
FeSO4.7H2O	27.8	27.8	27.8	27.8	FeH14O11S	27.800
Na2EDTA	37.5	37.5	37.5	37.5	Na2EDTA	37.300
Thiamine-HCl	2.5	10	10	2.5	Thiamine hydrochloride	0.100
Nicotinic acid	2.5	1	0.5	2.5	Nicotinic acid	0.500
Pyridoxine-HCl	2.5	1	0.5	2.5	PyridoxineHCl	0.500
Glycine	2	0	2	2	Glycine	2.000
Inositol	100	100	100	100	myo-Inositol	100.000
2,4-D	1	1	1	1	-	-
NAA	1	1	1	1	-	-
Kinetin	0.5	0.5	0.5	0.5	-	-
Maltose	30	30	30	30	Sucrose	30
Gelrite	2,700	2,700	2,700	2,700	-	-
Yeast Extract	100	0	0	0	-	-
Casein	0	2002	200	200	-	-

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Hydrolysate						
AgNO3	0	8	8	8	-	-
References	[35] [36] [37].					

148

149 At late uninucleate or early binucleate stage of microspore development were dissected under
150 sterile conditions. The culture anthers were inoculated onto a modified YP induction medium
151 (year reference) and supplements with 5 g/L charcoal, 500 mg/L tryptone, 120 g/L sucrose and 2
152 g/L gelrite at pH 5.8 and anther were incubated in the dark at 29 °C for 4 weeks [38]. [39] used
153 Modified YP without plant growth regulator, Modified YP +2 mgl-1kinetin, Modified YP +3
154 mgl-1kinetin, Modified YP +2 mgl-1kinetin+2 mgl-1 IAA, Modified YP +2 mgl-1kinetin+2
155 mgl-1 NAA and Modified YP +2 mgl-1kinetin+2 mgl-1 2-4-D media in their research. [40] used
156 MS medium containing, various concentrations of BAP, Kinetin, NAA (1-Naphthaleneacetic
157 acid) and 2,4-D (2,4- Dichlorophenoxyacetic acid) either alone or in combination. The cultures
158 were maintained at 25±2 °C and a 16 hours' photoperiod for four weeks. Different media were
159 used for regeneration and shooting is shown in table 1.2.

160 **Table 1.2**

161 **Table 1.2:** Regeneration and shooting media for haploid plant production

Regeneration median	**Composition**
MSB0	MSB with 30 g/l sucrose, 5 mg/l BA, 0.2 mg/l NAA, and 6 g/l agar
MSB1	MSB with 40 g/l sucrose, 5 mg/l BA, 0.2 mg/l NAA, and 6 g/l agar
MSB2	MSB with 40 g/l sucrose, 4 mg/l BA, 1 mg/l NAA, 5 mg/l adenine, and 6 g/l agar
MSB3	MSB with 40 g/l sucrose, 4 mg/l BA, 0.5 mg/l NAA, 10 mg/l adenine, and 6 g/l agar
XN	N6 with 40 g/l sucrose, 4 mg/l BA, 1 mg/l NAA, 5 mg/l adenine, and 6 g/l agar
R1	MSB with 40 g/l sucrose, 4 mg/l BA, 1 mg/l NAA, and 6 g/l a
Shooting media	**Composition**

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MR1	MSB (1/2 macrosalts content) with 30 g/l sucrose, 1 mg/l IBA, and 6 g/l agar
MR2	MSB (1/2 macrosalts content) with 30 g/l sucrose, 0.5 mg/l IBA, and 6 g/l agar
MR3	MSB (1/2 macrosalts content) with 40 g/l sucrose, 0.25 mg/l IBA, 0.25 mg/l NAA, and 6 g/l agar
MR4	MSB (1/2 macrosalts content) with 40 g/l sucrose, 0.25 mg/l IBA, 0.5 mg/l NAA, and 6 g/l agar
References	[41]

Chromosome counting:

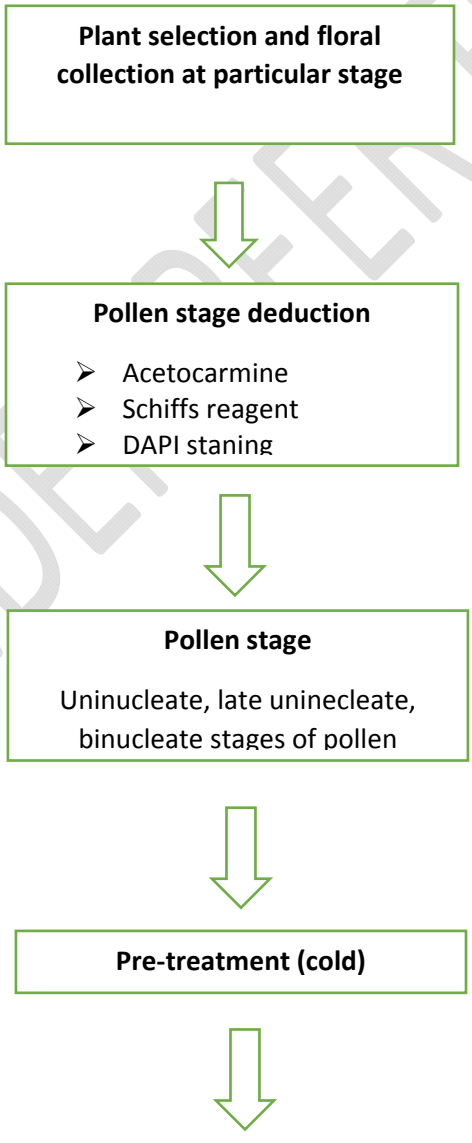
Methods for ploidy level determination in haploids and DH plants and can be identified by chromosome counting during meiotic cell division. Root tips or other meristamatic tissues can be used for chromosome counting during mitotic division [42]. Chromosome counting during mitotic division is time consuming and difficult in small chromosomes like *Brassica napus* [43].

Chromosome doubling by colchicine:

Haploid plants are short, infertile and sterile due to pollen or egg not able to produce pollen and egg because of absence of homologous pair in meiotic division. Chromosome double must be needed for the survival of haploid plant [44]. Colchicine, an alkaloid found in the seeds of autumn crocus (*Colchicum automnale*). It is an antimitotic agent and used for chromosome doubling, restored fertility of delete some sterile interspecific, intergeneric hybrids of plants, andro-and gynogenic haploids. Effective colchicine treatments have been reported for several crop plants like wheat, maize, rapeseed, Indian mustard, and watermelon [45]. Plant breeding programme needs doubled haploid for hybrid development [44]. Treatment of Colchicine can be applied on post pollination to the tillering stages of the haploid plants. In wheat and barley, colchicine treatment is normal suggested the 3- to 4-tiller stage, time 5–8 hours (treatment uniform) by submerging root system in a colchicine solution having 0.3 ml/l Tween 20, 2% dimethyl sulfoxide (DMSO), 0.1% colchicine, and 10 mg/l of gibberellic acid. Doubling rate can reach 95.6%. The colchicine can be practiced before the seedling stage of haploid plants by using 0.5% colchicine to the rescue medium for 48 h. The colchicine solution (1% colchicine with 100 ppm 2,4-D) can be injected into the uppermost internode of pollinated spikes at 48 and 72 hours after pollination, and the chromosome-doubling rate varies from 33% to 100%. After treatment, the plantlets were washed with running water, transferred to soil and having in a growth chamber

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at 14– 16°C (uniform temperature) under 16/8 h day/night before the new tillers emerged. The plants were shifted to normal conditions in a greenhouse. The plant survival rate reached 99% and the chromosome-doubling rate varies 96–98% [5]. Please check reference



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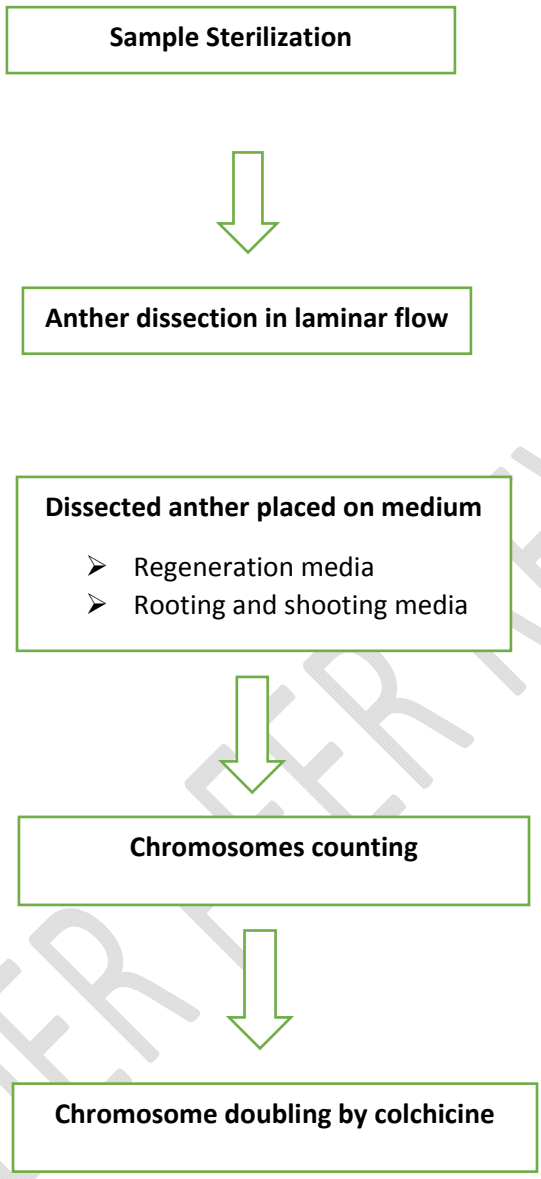


Fig 1; Diagram explaining the anther culture techniques

Implementations of Doubled Haploids

Double haploids production is very attractive tool in modern plant breeding. Many methods can be used for production of DHs, gynogenesis is used as a substitute method in plants where issue of male sterility is high [46]. Anther culturing has many advantages over classical techniques in the betterment of the crop plants [47]. Instant fixation of homozygous genes, improved selection,

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increased genetic variation and early expression of recessive genes are some important benefits of doubled haploids [15]. Following are delete some important uses of DHs explained by [48].

Uses in Breeding Crops:

1. DHs can be used to synthesize the homozygous lines or pure lines in self-pollinated crops and inbred lines in cross pollinated crops.
2. Additional and alternative lines explain can be produced.
3. Earlier release of variety is obtained as it reduces the time for achieving the homozygosity.
4. Development of homozygous lines/ As there is great similarity so no masking effect of genes.
5. Greater efficacy is observed because it is easy to combine targeted genes in homozygous line.
6. Selection is done in first generation of haploids because high selection pressure is applied at this stage.

Uses in Genomics:

Important applications of doubled haploid in field of genomics.

1. This can be used to recover the recessive genotypes.
2. Occurrence of mutation and its spectrum can be studied through doubled haploids.
3. The great help in mapping of population can be taken from doubled haploids.
4. It is reproducible and can be conducted at different sites explain.
5. The expressivity of a gene can be increased through DHs.
6. Genes can be located and tagged in the DH populations.
7. Traits can be selected through recognition of markers.
8. DH populations also help in Quantitative trait loci because authentic phenotyping of traits can be performed.

Doubled haploids are known for their extreme homozygosity and reduced time required for fixing desirable genes. The time needed can be reduced by 4 years by using DHs as compared with old breeding methodologies [49]. By using DHs the number of meiotic steps are reduced as

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compared to filial generations in getting homozygosity. DHs face less crossing over so they are contributed by the greater portion of the parental DNA than by single seed descent. It is indicating that DHs will inherit higher segment of DNA from parents than other methods [50]. DHs can be used where high parental proportion is required. According to Frisch and Melchinger (reference year) in quantity of 0.99 quantile SSD (single seed descent) method was able to recover 78% of parental genome while DHs progeny ensured higher proportion by showing 82% parental contribution. Hence doubled haploids in addition to reducing the time required is also involved in resembling more closely to parents than other methods. It can be referred as reverse breeding [51].

Conclusion and future horizons:

Haploid and doubled haploid breeding is a very fertile system of modern breeding. It can be the substitute of conventional breeding in which all steps are very laborious and time consuming in future. Anther culturing is a very effective technique that has been successfully used in many important plant species for the reducing time required to develop important genotypes and achieving extreme homozygosity. The plants like coffee require high time for regeneration such species can be subjected to these techniques and time span can be reduced. Although anther culture is responsive to a wide range of genotypes and gives satisfactory results but many important species are under considerations. Many other mechanisms involved in haploid breeding at molecular and biochemical level needed to be studied. In addition to this anther culturing needed to be cost effective, more efficient, practical and satisfying the breeding needs. Hence there is need of improvement of protocols to develop new genotypes. Modern fields of genetics have benefitted the micro embryogenesis. The advancements and research in this field will improve understanding of this process and lead to effective procedures development for embryogenesis in future. Further increasing number of facts and evidences shows the interest of future researchers in anther culture for double haploid production

Reference:

[1]. Tefera A. Review on concept and impact of double haploid techniques in crop improvement. Jour. of Natural sci. research. 2017; 7:23.

[2]. Gerald N, Frei U and Lubberstedt T. Accelerating plant breeding. Trends in plant science. 2013;18(12):667-672.

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[3]. Seguí-Simarro J. Doubled haploidy in model and recalcitrant species. Frontiers in plant science. 2015;6.

[4]. Croser J, Lülsdorf M, Davies P, Clarke, Bayliss L, Mallikarjuna N and Siddique H. Toward Doubled Haploid Production in the Fabaceae: Progress, Constraints, and Opportunities. Critical Reviews in Plant Sci.2006; 25(2): 139-157.

[5]. Niu Z, Jiang A, Hammad A, Oladzadabbasabadi A, Stevens, XU S, Merhoum M and Elias E. Review of doubled haploid production in durum and common wheat through wheat x maize hybridization. Plant breeding. 2014;133: 313–320.

[6]. Asif M. Progress and opportunities of doubled haploid production. Springer. 2013.

[7]. Germena M. Anther culture for haploid and doubled haploid production Plant. Cell Tiss. Organ Cult. 2010;104: 283–300. (fullstop)

[8]. Castillo A, Cistué L, Vallés M and Soriano M. Chromosome doubling in monocots. In Advances in Haploid Production in Higher Plants 2009;329-338.

[9]. Forster P, Herberle-Bors E, Kasha K and Touraev A. The resurgence of haploids in higher plants. Trends Plant Sci. 2007;12(8):368–375. (fullstop)

[10]. Alheit K. Rei J, Maurer H, Hahn V, Weissmann E, Miedaner T. and Würschum T. Detection of segregation distortion loci in triticale (x Triticosecale Wittmack) based on a high-density DArT marker consensus genetic linkage map. BMC genomics. 2011;12(1):380.

[11]. Wurschum T, Tucker M, Reif J and Maurer H. Improved efficiency of doubled haploid generation in hexaploid triticale by in vitro chromosome doubling. BMC plant biology; 2012;12(1):109.

[12]. Murovec J and Bohanec B. Haploids and doubled haploids in plant breeding. In Plant breeding. InTech 2012.

[13]. Wędzony M, Forster B, Żur I, Golemiec E, Szechyńska-Hebda M, Dubas E and Gotębiowska G. Progress in doubled haploid technology in higher plants. In Advances in haploid production in higher plants, Springer Netherlands. 2009;1-33.

[14]. Grauda D, Mikelsone A, Lisina N, Zagata K, Ornicans R, Fokinac O, Lapina L and Rashal L. Anther culture effectiveness in producing doubled haploids of cereals. Latvian Acad. Sci. 2014:68(2014):142-147.

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[15]. Mishra R, Rao G. In-vitro Androgenesis in Rice: Advantages, Constraints and Future Prospects. Rice Science. 2016;23(2):57-68.

[16]. Tripathy SK, Swain D, Mishra D, Prusty AM, Behera SK, Tripathy P and Chakma B. Elucidation of the genetic basis of anther culture response and its breeding perspective in rice. Euro. Jour. of Biotechn. and Biosc.2018;6(2): 26-30.

[17]. Mishra R., Rao GJ N. and Rao RN. Effect of cold pretreatment and phytohormones on anther culture efficiency of two indica rice (*Oryza sativa* L.) hybrids: Ajay and Rajalaxmi. J Exp Biol Agric Sci, 2013;1(2): 69–76.

[18]. Dunwell J. Haploids in flowering plants: origins and exploitation. Plant Biotechnology Journal. 2010;8(4):377-424.

[19]. Germanà M. Doubled haploid production in fruit crops. Plant Cell, Tissue and Organ Culture. 2006;86(2):131-146.

[20]. Germana M. Haploid and doubled haploids in fruit trees. In: Touraev A, Forster B, Jain M (eds) Advances in haploid production in higher plants. Springer, Heidelberg, 2009:241–263.

[21]. Dunwell J. Patents and haploid plants. In: Touraev A, Forster B. P, Jain SM (eds) Advances in haploid production in higher plants. Springer, Heidelberg. 2009:97–113.

[22]. Silva T and Ratnayake W. Anther culture potential of indica rice varieties, Kurulu Thuda and BG250. Trop Agric Res Ext. 2009:12(2): 53–56.

[23]. Al-Ashkar I. Anther culture response and salt tolerance in some wheat genotypes. Annals of Agricultural Sciences. 2013;58(2):139-145.

[24]. Sriskandarajah S, Sameri M, Lerceteau-Köhler E, Westerbergh A. Increased Recovery of Green Doubled Haploid Plants from Barley Anther Culture. Crop Science. 2015;55(6):2806.

[25]. Grewal R, Lulsdorf M, Croser J, Ochatt S, Vandenberg A, Warkentin T. Doubled-haploid production in chickpea *(Cicer arietinum* L.): role of stress treatments. Plant Cell Reports. 2009;28(8):1289-1299.

[26]. Cha-um S, Srianan B, Pichakum A and Kirdmanee C. An efficient procedure for embryogenic callus induction and double haploid plant regeneration through anther culture of Thai aromatic rice (*Oryza sativa* L. subsp. indica). In-vitro Cell Dev. Biol. 2009;45(2):171–179.

UNDER PEER REVIEW

[27]. Silva T. Indica rice anther culture: Can the impasse be surpassed? Plant Cell Tiss. Organ. Cult. 2010;100(1):1–11.

[28]. Bishnoi U, Jain R, Rohilla J, Chowdhury V, Gupta K and Chowdhury B. Anther culture of recalcitrant indica × Basmati rice hybrids. Euphytica. 2000;114(2):93–101.

[29]. Mohiuddin AK, Karim H and Sultana S. Development of improved doubled-haploids through anther culture of indica rice (*Oryza sativa* L.). Annals of Bio. Resear. 2014;5(10):6-13.

[30]. Ambarwati A, Somantri H, Utami W, Apriana A and Moeljopawiro S. Rice Anther Culture to Develop Double Haploid Population and Blast Resistant Lines. Jurn. AgroBio. 2011;5(2):71-77.

[31]. Min J, Sim S, Lee S, Park H, Park GH and Kim M. Comparison of the Efficiency to One-Step and Two-Step Anther Culture in Rice (*Oryza sativa* L). Jour. of Agri. and Envi. Sci. 2015;4(1):77-82.

[32]. Afza R., Shen M, Zapata J, Xie J, Fundi K, Lee S, Eva M and Kodym A. Effect of spikelet position on rice anther culture efficiency. Plant Sci. 2000; 153:155-159.

[33]. Roy B and Mandal B. Anther culture response in indica rice and variations in major agronomic characters among the androclones of a scented cultivar, Karnal local. Afr. Jour. of Biotech. 2005;4(3): 235-240.

[34]. Sen C and Singh P. Anther culture responses in boro rice hybrids. Asian Jour. Off biotech. 2011;3(5): 470-477.

[35]. Murashige T and Skoog F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physi. Plantarum. 1962;15.

[36]. Chu C. The N6 medium and its applications to anther culture of cereal crops. In: Proceedings of Symposium on Plant Tissue Culture, Science Press, Peking, 1978;45-50.

[37]. Kaushal L, Balachandran M, Ulaganathan K and Shenoy V. Effect of Culture Media on Improving Anther Culture Response of Rice (*Oryza sativa* L.). Intern. Jour. of Agri. Innov. and Resear. 2014;3(1): 2319-1473.

[38]. Jager K, Bartók T, Ordog V and Barnabas B. Improvement of maize (*Zea mays* L.) anther culture responses by algae-derived natural substances. South Afri. Jour. of Botany.2010;76: 511–516.

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[39]. Hosseini S, Ismaili A and Mohammadi P. Improvement of maize (*Zea mays* L.) anther culture embryogenesis and direct regeneration by different plant growth regulators and gelling agent. Jour. of Applied Biotech. Reports. 2014;1(1):17-21.

[40]. Madke S, Cherian J and S Badere. A modified Murashige and Skoog media for efficient multipleshoot induction in *G. arborea* Roxb. Jour. of Forestry Research. 2014; 25(3): 557−564.

[41]. Bobkov S. Obtaining Calli and Regenerated Plants in Anther Cultures of Pea. Czech J. Genet. Plant Breed. 2014; 50:123–129.

[42]. Dwivedi S, Anne B, Britt L, Tripathi S, Sharma, Upadhyaya D and Ortiz R. Haploids: Constraints and opportunities in plant breeding. Biotech. Advan. 2014;33: 812-829.

[43]. Weber S, Wilfried L and Friedt W. Efficient doubled haploid production in *Brassica napus* via microspore colchicine treatment in vitro and ploidy determination by flow cytometry. Weber-Lühs-Friedt GCIRC-Bulletinpp. 2004;1–6.

[44]. Das A,Kumar K, Kishor U. Tribhuvan, Das S and Mishra M.. Development of Haploid and Double Haploid in Fruit Crops - A Review. Internat. Jour. of Current Microbio. and Applied Sci. 2018;7(5): 2119-2132. (fullstop)

[45]. Burun B and Emlroglu U. A Comparative Study on Colchicine Application Methods in Obtaining Doubled Haploids of Tobacco (*Nicotiana tabacum* L.). Turk J Biol. 2008;32: 105-111.

[46]. Bhat J and Murthy G. Factors affecting in-vitro gynogenic haploid production in niger (*Guizotia abyssinica* (L. f.) Cass.). Plant Growth Regul. 2007;52(3): 241–248.

[47]. Purwoko B, Dewi I, Khumaida K. Rice anther culture to obtain doubled-haploids with multiple tolerances. AsPac J Mol Biol Biotechnol. 2010;18(1): 55–57.

[48]. Badu M, Tripathy B, Sahu G, and Jena A. Role of doubled haploids in vegetable crop improvement.Journal of Pharmacognosy and Phytochemistry. 2017;6(6): 384-389.

[49]. Smith S, Hussain T, Jones E, Graham G, Podich D, Wall S, Williams M. Use of doubled haploids in maize breeding: implications for intellectual property protection and genetic diversity in hybrid crops. Mol Breeding. 2008; 22:51–59.

[50]. Frisch M and Melchinger A. Variance of the parental genome contribution to inbred lines from biparental crosses. Genetics e publication. 2007

UNDER PEER REVIEW

[51]. Dirks G, Van Dun P and Reinink K. Reverse Breeding. Intl. Publ. No. WO 03/017753 A2. World Intellectual Property Organisation. Geneva, Switzerland. 2003;83.

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