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Development of Onion Hybrids using Cytoplasmic Genetic Male Sterility**

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ABSTRACT

Onion (*Allium cepa*) is an important vegetable/spice crop worldwide. It is an open pollinated crop; therefore, development of onion hybrids is most sought out option to boost onion productivity. Use of cytoplasmic genetic male sterility (CGMS) is most pragmatic method ofonion hybrid development. This includes use of three lines, *i.e.,* cytoplasmic male sterile line (A), its isogenic fertile maintainer line (B) and fertility restorer line (R). The conventional method of hybrid development is less efficient as compared to molecular marker assisted methods for identification of S, T, N cytoplasm, maintainer lines and fertility restorer genes with high heterosis. Various molecular marker systems have been established for commercial hybrid seed production. This review examines the development and identification of male sterile and its maintainer lines as well as restorer lines for hybrid seed production using conventional and molecular marker assisted methods.

Keywords**:** *Allium cepa*, hybrids, cytoplasmic genetic male sterility, molecular marker, restorer lines

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Introduction

Onion is one of the most important vegetable/spice crop worldwide used in vegetarian and non-vegetarian culinary. There are different types of onion depending on shape, size and colour. Onion is rich in fibre, Vitamin E, mineral matter and bioactive compounds and antioxidant properties (Ren and Zhou, 2021). Onion originated in Central Asia, Near- East and Mediterranean regions is a diploid species (2n = 16), belonging to family Alliaceae (Vavilov, 1951). Onion is grown mainly in countries of Asia, Middle east, Europe and North America. Onion is also an important vegetable crop in Eurasia including Turkey, Tajikistan, Kyrgyzstan and Uzbekistan. The major onion producing states in India are Maharashtra, Karnataka, Madhya Pradesh, Gujarat, Bihar, Andhra Pradesh, Rajasthan, Haryana, and Tamil Nadu. In India it is grown over 16.24 million hectare area with production 26.6 million tonnes and productivity 1.64 mt/ha in 2020-21(Agricultural Statistics at a Glance 2021).

Onion is a cross pollinated crop. In such crop, heterozygosity *per se* has to be maintained for better performance as selfing will lead to inbreeding depression and decreased performance. Therefore, the germplasm in onion is available in the form of open pollinated population and hybrids. Onion hybrids are known for their higher bulb yield potential as well as bulb quality. Flower of onion is perfect and tiny with 6 stamens and many flowers are arranged in umbel shaped inflorescence. Heterosis has been commercially exploited in onion since male sterility is available. The male sterility was reported in this crop long back in 1936 (cf Sidhu et al*.,* 2005). Honeybees are used for necessary transfer of pollen from male parent to female parent (Sidhu et al*.,* 2005). In conventional onion breeding, hand emasculation of onion flowers by removal of stamens is inefficient, time consuming,

labour intensive and costly proposition posing strong restriction on development of hybrid seeds to meet the demands of onion growers. Therefore, male sterility systems in onion are called for development of onion hybrids in an efficient manner. There are two strong possibility of male sterility system in onion: **1) Genetic male sterility-** This kind of male sterility is governed by dominant nuclear genes (Liu et al., 2019), which would segregate in cross population producing male fertile and male sterile plants. In such systems, male fertile plants need to be rouged out to develop onion hybrids using male sterile plant in the population. Rouging of male fertile plants essentially need markers, which are stable distinct and conspicuous to facilitate identification of male fertile plants before flowering. Due to this restriction genetic male sterility is hardly used in commercial onion hybrid seed production.

The 2nd option is based on cytoplasmic genetic male sterility systems which involve three lines *i.e.* cytoplasmic male sterile lines (A line), itsisogenic maintainer line (B line) and fertility restorer line (R line) yielding maximum heterosis/hybrid vigour in F_1 generation of crosses. This is most commonly used method for production of onion hybrid seeds. The future scope of commercial onion hybrids needs to be focused comprehensively for the identification of male sterile lines from onion open pollinated population by utilizing modern biotechnological tools. Molecular markers distinguishing sterile/fertile cytoplasm (N, S, T) linked to restorer (Rf) of male sterility locus (*Ms*) are important. Release of commercial hybrids from public/private sector would play a great role for breaking yield barriers and significant enhancement of productivity of onion under changing climate scenario and to meet increasing domestic demand (Singh and Khar, 2021). This review paper examines the important aspects of cytoplasmic male sterility system, procedure for development of onion hybrid and production of hybrid seeds using conventional and molecular marker assisted methods.

Cytoplasmic-Genetic Male Sterility System:

Male sterility is defined as a conditions of the flower where the anthers' are either missing or the pollen grains are non viable. Therefore, self pollination in complete male sterile lines is ruled out. Sidhu et al., (2005) reviewed male sterility in onion. According tothem it was discovered as early as1925 from Italian Red-13- 53, the causes of male sterility in this selection were investigated in 1926 and the use of male sterility systems for hybrid seed production were reported in 1943. Davis (1957) accounted the distribution of male sterility gene in different onion varieties. Banga and Petiet (1958) pointed that Dutch cms-T line had been isolated as point mutation from the line containing N-cytoplasm. Peterson (1970) utilized cytoplasmic male sterility (CMS) in the development of hybrid onion and suggested that recycling of selected onion inbreds' and selection for combining ability and quality be undertaken early. He isolated an open-pollinated single plant progeny with 21% higher yield than original cultivar. Gikalo (1972) noted morphological differences in umbels and flowers of normal and male sterile plants. He described a method of overcoming inbreeding depression in normal and male-sterile inbreds' by pollination with self-pollen from normal plants of same variety and multiplied them further.

Kazakova and Yakovlev (1973) developed 20 male sterile lines and 98 hybrid combinations with male sterile line Oriental S57, Oriental S61, Golden Globe S1 Valencia S1, Bessonovka S36 and Poyar, which exhibited heterosis for earliness, storage quality and yield of marketable quality onion. Dyki (1973) studied CMS in onion varieties Wolska and Rawska and noted abnormalities in male-sterile plants as withered stamens couples with long pistils, glossy looking green anther containing only watery fluid. Microscopic investigation confirmed 100% male sterility in lines. In fertile hard, brown anther supported on normal filaments produced fertile pollen grains. Holford et al., (1991a; b) observed difference in both chloroplast and mitochondrial genomes of N and S cytoplasm. However, no differences were detected in organellar genomes of N and cms-T onions suggesting different auto- and allo-plasmic origins of cms-T and cms S cytoplasms. Havey (1993) reported five polymorphisms between S sterile and N fertile cytoplasms where former was different form *Allium* species closely related to bulb onion. Havey (2000, 2004) studied diversity among CMS that provides an expedient mechanism to produce large population of male sterile plants for commercial F_1 hybrid seed production.

Melo and Boiteux (2001) attempted molecular identification of male-sterile line (line A) and maintainer (line B) but found only fertile cytoplasm in the Alfa Tropical population (Leite, 1999). However, lines A and B within the BaiaPeriforme derived onion population, Alfa Sao Francisco, based on a PCR marker system monitoring cytoplasm type, and by random field pairing of fertile plants with selected sterile plants, appeared important to develop tropical onion hybrid well-adapted to Brazilian low latitudes.

Yamashita and Tashiro (2004) developed male-sterile lines of Japanese bunching onion (*A. fistulosum*) possessing the cytoplasm of wild species, *A. galanthum*, by backcrossing. Yamashita et al., (2005) confirmed that the fertility-restoring gene (Rf) for

cytoplasmic male-sterility (CMS) in *A. fistulosum* from segregation of pollen fertility of backcross generation of *A. galanthum* is located on the 5F chromosome of the male fertile plants.

Work on hybrid seed production of onion using cytoplasmic male sterility was examined and the problem of protecting lines from genetic contamination and maintaining their high values for useful characters was described Khaisin (1988). Pathak and Gowda (1994) inferred that one of the main components for the exploitation of heterosis was the availability of male sterility. Indigenous male sterility was found in *cv.* Nasik white globe at Bangalore, having strong cytoplasmic factor for male sterility. It was successfully transferred to six different genotypes which were used to exploit heterosis. From 75 test crosses evaluated for bulb yield and quality, two were promising, Hybrids (Ms 65 x Sel.13) and hybrid–5 (MS-48 X Sel.14) for commercial use, with high bulb yields (45-50t/ha) and good quality bulbs. Pathak (1997) identified a second source of cytoplasmic male sterility (T-cytoplasm) with complex inheritance which was different form Jones's line with three independent segregating restorers. Havey (2006) reported two different sources of CMS (S and T cytoplasms). Test crosses of N-cytoplasmic maintaining and restoring genotypes to S and T cytoplasmic lines demonstrated that different alleles, or loci, restore male fertility for these two male-sterile cytoplasms. Other sources of CMS have been used or reported in Europe, Japan and India, and their relationships to S and T cytoplasms are still not clear. Restriction fragment length polymorphisms were identified in the organellar genomes among commercially used male-sterile cytoplasms from Holland, Japan and India, and were compared to S and T cytoplasms. Mitochondrial DNA diversity among 58 non-S-cytoplasmic open pollinated onion populations was also assessed. All five putative CMS lines selected from the Indian population Nasik White Globe were identical to S cytoplasm for all polymorphisms in the chloroplast genome, and always possessed the same-sized mitochondrial fragments as S cytoplasm. T cytoplasm, the male-sterile cytoplasm used to produce the Dutch hybrid Hygro F_1, and two sources of CMS from Japan, were similar and showed numbers of mitochondrial polymorphisms similar to those observed among the 58 non-S-cytoplasmic open-pollinated populations. This research demonstrated that the same, or very similar, male-sterile cytoplasms have been independently isolated and exploited for hybrid-seed production in onion.

Haishan et al., (2006) reviewed characterization and utilization of the S and T types of male sterile cytoplasm of onion. Netrapal et al., (1986) screened large number of onion lines to isolate the male sterile lines from a popular short day variety 'Pusa Red'. The maintainer lines were developed by backcrossing. Sharma (2018) reported isolation and identification of male sterile line from variety Hisar onion 2. He reported two methods for identification and validation of male sterility which included the touching sense of onion flowers at full bloom. The male sterile plants were oppressed as the anthers' were shrunken being devoid of pollen grains. The second method was based on pollen staining with acetocarmine stain and the stained pollen grains were observed under microscope. The flowers giving pink coloured pollen grain were considered to be male fertile and non-coloured hayeline pollen grains were non-fertile. Sharma (2022) reported 8 male sterile lines isolated from Hisar onion 2 and used these lines to produce F_1 hybrids.

Molecular basis of Cytoplasmic-Genetic Male Sterility System:

Cytoplasmic Genetic Male Sterility is governed by genetic mechanisms, which are influenced by the environment for its final expression. The ambiguity caused by environmental factors may sometimes jeopardise the whole effort of developing onion hybrids based on male sterility. Therefore, it is essential to target genetic mechanisms determining male sterility then the phenotypic expression for precision and efficiency.

Three commercially used sources of onion CMS can be distinguished by markers in the cp and mt DNAs. S and R cytoplasms which were commonly used sources of onion CMS; T cytoplasm as described by Berninger (1965) were rarely used commercially. N and T cytoplasms could be distinguished by mitochondrial polymorphisms cob and orfA501 developed by Engelke et al., (2003). Cho et al., (2005) selected N- cytoplasm plants using sequence characterized amplified region (SCAR) marker from 'Manchuhwang' (open-pollinated cultivar). Selected N-cytoplasm plants were crossed with male sterile inbred line (W202A, Wisconsin Univ., USA). A total of 66 crosses were accomplished, and 34 crosses could be analysed for the nuclear restore allele. Among 34 combinations, the offspring's of one combination showed all male sterility and this line was selected as the maintainer line in the 'Manchuhwang'

Kim et al., (2007) applied the PCR-marker (orfA-501) to identify the cytoplasmic genotypes of collected 100 accessions of bulb onion. Among accessions, S-cytoplasm was found in 57 accessions. Nineteen accessions possessed only N-cytoplasm and twenty

four accessions possessed both S- and N-cytoplasm. Two sets of cytoplasmic male sterile lines from two different onion cultivars (*Allium cepa* L.), were investigated by molecular method, and discussed about their polymorphisms with corresponding maintainers. The results showed that the cytoplasms of male sterile lines originated from 'ShagouHongpi' and 'ShuozhouZipi' were T and S, respectively. RAPD amplification showed that the S cytoplasm had more polymorphism than T cytoplasm compared to their maintainer lines (Jingfan, 2009).

R cytoplasm could be distinguished from the other cytoplasms by the presence of both 628- and 833-bp amplicons of mitochondrial orf725 (Kim et al*.,* 2009a). S cytoplasm could be distinguished from N, R, and T cytoplasms by chloroplast markers (as reviewed by Kim et al., 2015b). With these cytoplasmic markers, onion breeders should be able to confidently determine cytoplasms in commercial use. Although they did not score male fertility *vs.*sterility across the onion inbreds used in their study, genotypes at *Ms* as predicted by the AcPMS1 marker (Kim et al*.,* 2015a) were consistent with previous reports of male-fertility restoration by dominant allele(s) at the *Ms*locus for S and R cytoplasms Kim (2014), both of which produce the orf725 amplicon.

Ferreira et al., (2017) used molecular marker to identify the cytoplasmic types and the genotyping for the fertility restoration nuclear locus (*Ms*) in 59 onion accessions, aiming at the selection of 'A' and 'B' lines. Three markers were used to identify the cytoplasm 5' *cob*, *orf*A501, and *orf*725, and two were used for the *Ms* locus (AcSKP1 and AcPMS1). The two types of male-sterile cytoplasm ('S' and 'T'), as well as fertile cytoplasm ('N'), and the *Ms* and *ms* alleles in both homozygosity and heterozygosity were detected in the 59 genotypes. The frequencies of the 5' *cob*/*orf*A501 and *orf*725 markers, as well as of the markers AcSKP1 and AcPMS1, were close in the onion accessions. In the Brazilian germplasm, the frequencies of the 'N', 'S', and 'T' cytoplasm were approximately 0.47, 0.28 and 0.25, respectively, whereas the allele frequencies of *Ms* and *ms* were 0.52 and 0.48, respectively.

Identification of male sterile lines through conventional methods require 4-8 years of progeny testing before the cytoplasm type can be determined. Gazendam et al., (2018) analyzed five cytoplasmic (5'cob, orfA501, orf725, IGS and cob-type 2) and four nuclear markers (jnurF13, isotig34671_610, isotig30856_1351 and isotig29186_1830). Real-time polymerase chain reaction (PCR) was performed with custom TaqMan® SNP genotyping assays containing primer/probe pairs designed to detect single nucleotide polymorphisms (SNPs) linked to the nuclear *Ms* locus. OrfA501 proved useful as a presence/absence marker for cytoplasmic male sterility, while TaqMan® SNP genotyping assays were superior to the jnurF13 nuclear marker in terms of rapid throughput. PCR molecular markers and custom TaqMan® SNP genotyping assays were efficient in screening the onion lines rapidly and accurately for their cytoplasmic and nuclear male sterility genotype. These methods reduced the time to identify the correct genotype of male sterile and maintainer lines and also gave accurate information for larger scale use.

Numerous studies have documented polymorphisms in the organellar DNAs differentiating S and T cytoplasms from the normal male-fertile cytoplasm of onion. There may be additional source(s) of onion CMS that had been described as "T-like" and appear to bemore similar to N and T cytoplasms than S cytoplasm. He also evaluated commercial identities of onion breeding lines using, molecular markers distinguishing sources of onion CMS. He reported that bona fide T cytoplasm is rarely used commercially to produce hybrid-onion seed, and both S cytoplasm and "T-like" cytoplasm were widely used. Dehghani et al*.,* (2021) evaluated the effectiveness of marker-assisted selection (MAS) in identification of the cytoplasmic types and male sterility (*Ms*) locus in 123 onion accessions. Three cytoplasmic markers cob, accD and MK were used to identify the sterility (S) from the fertility (N) cytoplasm and four nuclear molecular markers (OPT, PsaO, Jnurf-13 and AcSKP1) were used for genotyping of *Ms* alleles. The results showed that the two accD and cob markers were quite similar in the detection of the type of cytoplasm with 100% male sterility for male sterile lines and 100% fertility for maintainer lines. Also, the MK marker was able to distinguish T-type cytoplasm. The frequency of fertile (N) was much more than the frequency of sterile (S and T) cytoplasm found to be 90% in Dorche (pop.1), 100% in Dorche (pop.2) and Kashan based on marker cob and accD. With MK marker, it was found to be 80, 90 and 82% in Dorche (pop.1), Dorche (pop.2) and Kashan, respectively. Molecular markers were very suitable for the identification of S or N lines. Cytotype (N/S) determination of plants by using molecular markers (cob, accD and MK) and it could reduce the population size required for the production of onion hybrid seeds.

A process for identification of specific onion cytotypes was recently proposed by Havey and Kim (2021). They replaced the orfA501 marker (Engelke et al., 2003) with a new molecular marker, orf219, to improve the robustness of onion cytotype screening. The orfA501 marker is a dominant marker whose

genotypes are determined based on the presence or absence of PCR products. Kim et al., (2009b) reported that CMS-R and CM-S were widely used in onion hybrid breeding in Korea. In contrast, only two Korean breeding lines possessing CMS-T cytotype were identified. In the case of CMS-Y, only two accessions (PI273626 and PI236025) were shown to contain this cytotype. Progenies is derived from PI273626 showed unstable male sterility when this cytoplasm was combined with a dominant *Ms2* allele (Kim et al., 2019; Yu and Kim, 2021), breeding materials containing the CMS-Y cytotype may not be suitable for hybrid breeding. The CMS-T cytotype was identified in a limited number of accessions, which were mostly introduced from European countries Likewise, the CMS-T cytotype was identified from a single breeding line in a previous study (Havey and Kim, 2021), indicating that the CMS-T cytotype is rarely used in hybrid breeding. Given that inheritance of fertility restoration of CMS-T cytotype is relatively complicated, breeding materials containing this cytotype may be undesirable in hybrid breeding programs. The improved process developed in this study can be used to identify accessions containing CMS-T cytotypes. In contrast, male sterility conferred by CMS-S and CMS-R can be restored by a dominant allele at a single nuclear locus, Ms (Jones and Clarke, 1943; Kim, 2014), and molecular markers tightly linked to the Ms locus have been developed (Kim et al., 2015a; Kim and Kim, 2019). Therefore, breeding lines containing either CMS-S or CMS-R may be most beneficial for efficient development of hybrid onions.

Heterosis

The term heterosis was first coined by Shull (1914) as the developmental stimulus resulting from the union of different gametes, whereas, hybrid vigour referred to the manifest effect of heterosis (Whaley, 1944). Singh, 2021 has extensively reviewed the various aspects of heterosis including genetic mechanisms, dominance and super dominance theory of heterosis, heterozygosity advantage, hybrid vigour, prediction of heterosis in hybrids and inbreeding depression.

Kozlova (1963) noticed considerable increase in yield of some F_1 hybrids; out of 11 hybrids tested, yield increases ranged from 71 to 158 per cent over the female parent and 53 to 153 per cent over the pollen parent. Out of the 85 F_1 hybrids studied by Poljanstrij (1963) only three produced heterosis for yield. The F_1 hybrids from inter varietal yellow x purple crosses were intermediate in dry matter content, monosaccharides, polysaccharides and the ratio between disaccharides and monosaccharide's. The results of observation by Orlova (1969) revealed that among 60 F_1 hybrids, 22 exceeded the better parent, 10 were worse than both the parents and 28 were intermediate for yield. The most hybrid combinations were more vigorous and had larger number of leaves than the parents. Heterosis for dry matter content and sugars exceeded the better parent in two out of three F_1 hybrids studied by Genejdi (1970). Out of the 28 F_1 hybrids evaluated by Doruchowski (1971), four exceeded the Polish cultivars Wolska and Rawaska and the American hybrid Elite significantly in the yield of marketable bulbs by up to 40 per cent. Dry matter content was highest in hybrid with the highest yields, which gave 410.7 q/ha of marketable bulbs with 13.73 per cent dry matter. The F_1 hybrids also proved superiorin respect of yield, bulb size, plant survival, earliness, uniformity and yield of soluble dry matter in the direct seeded crop (Andrasfalvy, 1971). Total soluble solid is an important quality attribute of onion genotypes with high solids store better. McCollum (1968) and Padda et al., (1973) obtained high heterosis for total soluble solids in onions.

Joshi and Tandon (1976) showed a wide range of heterotic values for bulb yield (ranging from -22.1 to 72.3 percent over the average of the parents and from -42.8 to 36.7 percent over better parent) in crosses of four male sterile lines with five selections.

Hosfield et al., (1977) observed significant heterosis for bulb weight over mid parent. However, the heterosis over better parent was significant, but comparatively low. The work of Ershov and Vorobeva (1979) revealed that the F_1 hybrids using a cytoplasmic male sterile parent out yielded the standard by up to 55 per cent in ware (total) yields and by up to 109 per cent in the marketable yields. They further noted that the male sterile line 1 x 8 derived from Mstera, when used in the F_1 hybrid production, resulted in heterotic effect of 164 to 173 per cent regardless of pollinators used. Pandian and Muthukrishnan (1979) recorded heterosis for plant height, number of leaver, bulb weight, dry matter and total soluble solids, especially in crosses C8 856-8 x CO 1 and C8 665-51 x CO 1 of aggregation onion (*Allium cepa* L. var. *aggregation* Don.). In a diallel cross of onion at Indian Agricultural Research Institute, New Delhi, Netrapal (1980) reported up to 28.8 per cent of heterosis for yield over best parent. He also found that these F_1 hybrids were also superior for processing and storage qualities. Vadivel et al., (1981) observed fourteen hybrids over the mid parent and twelve hybrids over the better parent heterosis for plant height and high heterosis for bulb weight in onion.

Vadivel et al., (1982) evaluated 30 hybrids and their parents. The results showed both positive and negative heterosis for bulb weight, whereas, one cross recorded significant heterosis for yield and yield components.

Doruchowsk (1986) crossed eight male sterile lines with 8 pollen parents and heterosis was observed for bulb weight only. Madalagiri (1983) recorded significant positive heterosis over mid parent in six crosses and two crosses have exceeded their better parental value in a 9 × 9 diallel analysis for number of leaves and negative significance for TSS. Netrapal et al., (1988) observed only one hybrid with heterosis over both the better and best parent out of 29 crosses.

Aghora (1985) reported that study on heterosis using 20 lines and 3 male sterile lines as testers. Heterosis over the best parent was observed for all the characters except dry weight of leaves. Thirty five hybrids were heterobeltiotic for total bulb yield with highest heterosis of 89.56 per cent in the cross MS 39 x IHR 78. Positive heterosis was observed in nine hybrid over better parent for total bulb yield and it ranged from 47.9 to 89.5 per cent while heterosis over best parent for marketable bulb was over 35 per cent in three hybrid *viz*., MS-1 x NEK-1, MS-1 x IIHR-21-1 and MS-8 x IIHR-52-1(Pathak et al., 1987). Netrapal and Singh (1986) through a diallel analysis, recommended heterosis breeding for improvement of number of leaves, plant height, bulb-yield, bulb weight, bulb diameter (both polar and equatorial) and maturity.

Netrapal (1988) observed significant heterosis over better parent for number of leaves, plant height, maturity, yield, bulb weight, bulb diameter (horizontal and vertical), total insoluble solids, total soluble solids, total solids, drying ratio, pyruvic acid, reducing sugar, total sugars, non-reducing sugar and storage losses. Number of leaves, plant height, yield, bulb weight, bulb diameter, total soluble solids and storage losses showed heterosis over the best parent. Netrapal et al., (1988) reported that heterosis for plant height in 30 hybrids significantly exceeded the better parent, 18 exceeded the top parent and 44 exceeded the standard control. However, 8 hybrids were significantly superior to the better parent and 11 to the standard control. Gowda (1988) concluded that the extent of heterosis estimated over mid, better and best parents revealed superiority of some outstanding F_1 hybrids over best parent; for equatorial and polar diameter of the bulb. There were 16 crosses that exceed in heterotic effects over best parent for dry weight of the bulb.

Kumar and Dhaliwal (1990) described heterosis over mid and better parent value as well as standard check variety. Aghora and Pathak (1991) reported significant positive heterosis for number of leaves over mid parent in five crosses, whereas only one F_1 recorded significant positive heterosis over the better parent.

Sayed et al., (1999) evaluated the parents and F_1 hybrids from half diallel cross of onion for earliness, bulb ratio (neck diameter or bulb diameter), number of leaves per plant and bulb height. They observed highly significant additive and non-additive gene effects, which were involved in the inheritance of all the characters. Divakar (2001) reported positive heterosis in 12 crosses for total soluble solid over better parent and 10 crosses exhibited heterosis over standard check. Shashikanth et al., (2007) reported appreciable amount of heterobeltiosis and standard heterosis for marketable bulb yield. Satyanarayan (2014) reported heterosis for plant height, number of leaves, bulb weight, total bulb yield, bulb diameter (polar and equatorial) and TSS.

Sharma (2018) made a series of crosses between male sterile and restorer lines and found that the extent of heterosis over mid and better parent was variable from cross to cross and hybrid to hybrid. Sharma (2022) reported that the cross MS 35 x Hisar- 3, MS 37 x Hisar-3, MS 22 x Agrifound Dark Red, MS 40 x Pusa Red and MS 21 x Pusa Red exhibited high SCA as well as heterosis, hence, these crosses can be utilized for breeding heterotic hybrids.

Development of onion hybrids using CMS

1) Conventional methods

Sharma (2018, 2022) out lined the procedure for developing onion hybrids in field condition of Northern India. The development of hybrid involved mainly three phases, *i.e.,* selfing of open pollinated population to develop both cytoplasmic male sterile line and their maintainer as well as potential restorer lines to develop F_1 hybrids through crossing. These are detailed below:

Selfing: Selfing in onion is done only on a limited scale as it becomes difficult to maintain the inbred lines beyond S_2 generation due to drastic inbreeding depression. Selfing is done by putting individual cages over the plants. Flies were used to ensure pollination within cages. 2-3 umbels of the same plant were caged muslin cloth bag before anthesis. After anthesis, the umbels were rubbed against each other daily for a few days to ensure self-pollination.

Crossing: As soon as few buds in an umbel opened, the whole umbel of the female parent was bagged with muslin cloth bag. The opened flowers were removed daily for a few days to avoid selfing. The emasculated buds were retained for crossing. The umbel of pollen parent covered with muslin cloth bagswere cut off and its stalk were placed in a glass bottle filled with water and fastened to a bamboo/ wooden stake fixed in soil close to the female parent. Female parent umbel (emasculated one) and the pollen parent umbels are now enclosed in to each of the same common bag. Within a few days in the morning, male

umbel was gently rubbed over the emasculated umbel to ensure pollen shedding and cross pollination.

Use of male sterility:

The variety used for selecting male sterile line was Hisar Onion 2. Hisar Onion 3, Pusa Red, and Agri-found Dark Red used as tester line for hybrid development. Selfing and inter crossing activities were carried out in seed to seed planted crop following Jones and Mann (1963). The procedure of development of onion hybrid seed included following steps:

A. Isolation and maintenance of male-sterile line

1. Screening for male sterile plant in Onion Hisar-2 variety was planted in one acre area. Screening was done through morphologicalexamination with hand touching method.
2. Plants were categorized based on presence/ absence of pollen for occurrence of male sterility.
3. Pollen viability of twenty sampled male fertile plants were accounted under microscope with the help of acetocarmine 0.5% solution. Viable/fertile pollen showed pink colour and vigorous.
4. Pollen sterility of identified male sterile plant was accounted and confirmed under microscope with the help of acetocarmine solution 0.5% solution. Sterile pollen showed colourless and shrinked.
5. Flower umbels of selected *MS* (male sterile) plants were paired tied with umbels from fertile pollen donor plants.

i. 60 male sterile plant was isolated from Onion Hisar-2 and these male sterile plant pair tied with fertile (pollen donor) plants.

ii. All possible crosses (95 crosses) made with sterile and fertile plant.

6. Flower umbels of *MS* plants were paired tied with umbels of male fertile for hybrid development.
7. Basal ends of pair tied fertile umbels were placed in water filled bottles with close to *MS* plants.
8. Pair tied umbels, protected by muslin cloth bags, were shaked each morning for pollination.
9. Umbel spikes of fertile pollinator plants were covered in netted cages or muslin cloth bags for self-seed production.
10. Seed from male sterile and male fertile plants were harvested separately for next planting.

B. Identification and maintenance of maintainer line

1. Plant progenies raised from seed harvested in previous year were screened to confirm male sterility. F_1 progenies and their pollen parent raised separately in nursery during last week of July and seedling are transplanted in October with spacing of 45 x 45 cm row to row and plant to plant distance. Pollen donor parent transplanted in pair with their progenies and the recommended package of practices was followed to raise a healthy crop. During initiation of bolting plants were covered with netted cages or muslin cloth bags. At the time of dehiscence each plant was morphologically scored for sterile check and fertile status on the basis of pollen present in the anther by hand touching. Plant showing the presence of greenish yellow or yellowish brown or yellow pollen were designated as fertile and those without pollen as male sterile. After that, pollen sterility was accounted and confirmed under microscope with the help of acetocarmine solution.
2. Plant progenies were categorized based on proportion of male sterile and fertile segregants. Among 95 crosses only 8 crosses (MS20 x pollinator 5, MS21 x pollinator 5, MS22 x pollinator 5, MS23 x pollinator 5, MS34 x pollinator 11, MS35 x pollinator 11, MS37 x pollinator 11, MS40 x pollinator 11) showed 100% sterile plant.
3. Pollen donating plants showing cross progenies as 100% sterile were marked as maintainers.
4. Seed of maintainer, fertility restorer and parental line of hybrids were taken separately and saved.

Mohsin et al., (2016) in a field experiment identified one cytoplasmic genetic male sterile line (Smsms) and two fertile lines as maintainer lines (Nmsms). These two crossed materials namely 004 (Shallot x Red creole) and 008 (Shallot x Red pinoy) produced 100 per cent male sterile progeny infull sib and backcross generations. The Shallot x Taherpuri/ Suksagor and Shallot x Hazera-202/Hazera-203 cultivar produced both male fertile and male sterile segregating progenies. It indicated that these materials areprobably determined by dominant and recessive independently acting genes, which was resulting the genetically impure lines. All other crossed materials produced 100 per cent male fertile progeny upon crossing with shallot. Thus, the materials Red creole and Red pinoy could be used as maintainer line for "Shallot". The performance of 904 F_1 and 905 F_1 hybrids over check (Taherpuri) and better parent was found to be preferably better using these CMS system. They also outlined procedure for developing onion hybrids in field conditions which is detailed below in Figure 1.

2) Development of onion hybrids using molecular marker assisted selection for CMS, maintainer and restorer lines

Now a day molecular marker facilitated methods for identification of cytoplasmic male sterile lines (A line, CMS), their maintainer lines (B line) and fertility restorer line (R line) to be involved in crossing programmes to developed commercial onion hybrid (F_1) seeds.

Manjunathagowda (2021), reviewed use of molecular markers for identification of male sterile line, maintainer lines (N smsm) and homozygous fertility restorer line (NMsMs) as well as male sterile line (S msms) from various populations. He demonstrated use of cytochrome-b(cob) protein mitochondrial DNA marker as well asphenotypic examination to validate isolation of male-sterileand maintainer lines from open pollinated population. Malik et al., (2017) reported that the frequencies of male-sterile plants (Smsms) were 0.015 in Punjab Naroya, 0.020 in Punjab Selection and 0.006 in Punjab White. Whereas frequencies for the male sterility maintainer plants (Nmsms) were 0.133 in Punjab Naroya, 0.231 in Punjab Selection and 0.182 in Punjab. These frequencies were determined using cob marker. Ferreira et al., (2017) determined in Brazilian onion germplasm, frequencies of CMS-S, CMS-T and N cytoplasms which were found to be 0.47, 0.28 and 0.25 whereas, the Ms and ms allele frequencies were 0.52 and 0.48, respectively, using cob, orfA501 and orf725 genes-specific markers, and Ms/ms allelic markers of AcSKP1 and AcPMS1 genes for MAS. The frequency of male-sterile plants ranged from 0.77 to 0.80 across the open-pollinated populations, the significant number of male-sterile lines was noted in the genotypes COHBONC03 (6.5%), COHBONC05 (8.5%), COHBONC17 (4.0%) and COHBONC25 (5.5%) and thus identified male-sterile plants were confirmed by orf725 gene molecular marker (Manjunathagowda and Anjanappa, 2020).

Ahmad et al., (2020) identified the fertility-restorer locus (Ms) using two sets of nuclear markers (novel chimeric gene, orf725 gene (N/S) MK marker) and SCAR markers (FN1, RN1, F3S2 and R3S2) for Ms and ms allelic plants among the open-pollinated varieties (OPVs). It was found that, 70% of OPVs have male-sterile cytoplasm with recessive alleles at restorer-of- fertility (Ms) locus are male sterile (A-line), and nearly 20% of plants with normal (N) cytoplasm have recessive alleles (ms) at Ms locus (male sterility maintainer, B-line). Thus, the identification of A-, -B and R/C (restorer line) lines from OPVs aids for development for high-yielding F_1 hybrids in onion with the low production cost of hybrid seed. A schematic presentation of MAS based identification of three lines involved in hybrid seed production in onion is given below in Figure 2.

Future Out Look

Male sterility facilitated development of onion hybrid is both economical as well as quick method. Molecular markers have further accelerated the development onion hybrids over space and time in different country globally by precise identification of cytoplasmic male sterile line (CMS-S and CMS-T) male fertile maintainer line (Nmsms) and nuclear-male- fertility restorers (NMSMS) lines.

Discovery of these markers have opened up new avenues to harness hybrid vigour/heterosis through marker assisted selection (MAS) in onion. Various studies (Manjunathagowda, 2021) have revealed that molecular markers can be usedeffectively and profitably to speed up development of F_1 hybrids and production of pure hybrid seeds through MAS. Identification of male sterility locus (*Ms* locus) in background of different cytoplasms (S, T) is cumbersome and ambiguous is short day onion population. This can be achieved effectively through use of molecular markers to supplement and speedup conventional way of onion hybrid development methods particularly for selection of three lines in open pollinated populations with linkage disequilibrium for *Ms* locus. Onion breeders in future would be required to develop polymorphic codominant molecular markers (SSR, SNPs) for mapping genetic variation in base population and identification of potential lines for further breeding programme. Although, onion is one of the most important vegetable/spice crops worldwide with great economic end use significant, yet the pace of progress in developing MAS is far behind the cereal, pulses and other vegetable crops like potato and tomato. Onion breeders should conversion on use of modern *Omic* methods including genomics, transcriptomics, proteomics and metabolomics for precise and quicker identification of A, B and R lines at molecular levels to resolve various complexities in view of socio-economic importance of *Allium* crop. Germplasm collection from different onion production locations worldwide and its characterization through genetic analysis and molecular markers is imperative to speed up the genetic improvement in this crop for sustainable production under climate change to meet the growing demand of consumers at national and international levels.

The most fascinating feature of onion hybrid breeding programme would be targeted to development of inbred lines through haploid induction as well as to fix heterosis in onion hybrids using double haploid approaches. Use of apomixes can also be explored for achieving this objective.

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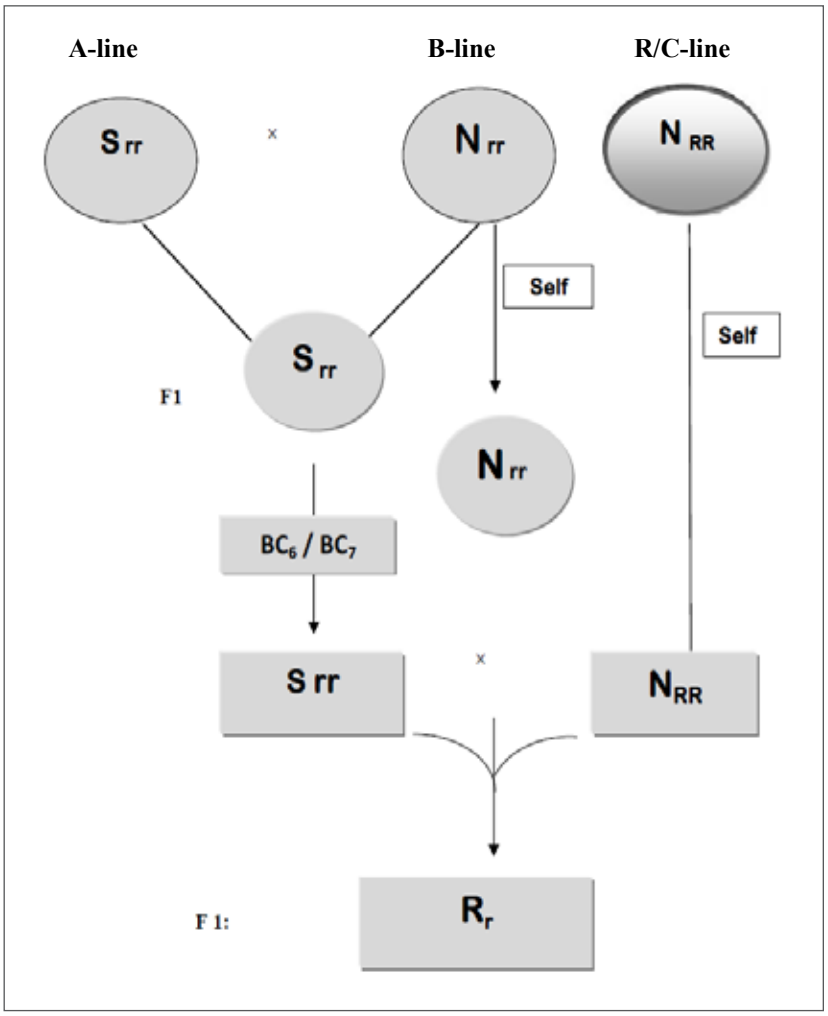

Figure 1. Flow diagram of various steps involved in development of onion hybrids using CMS system (Mohsin et al., 2016).

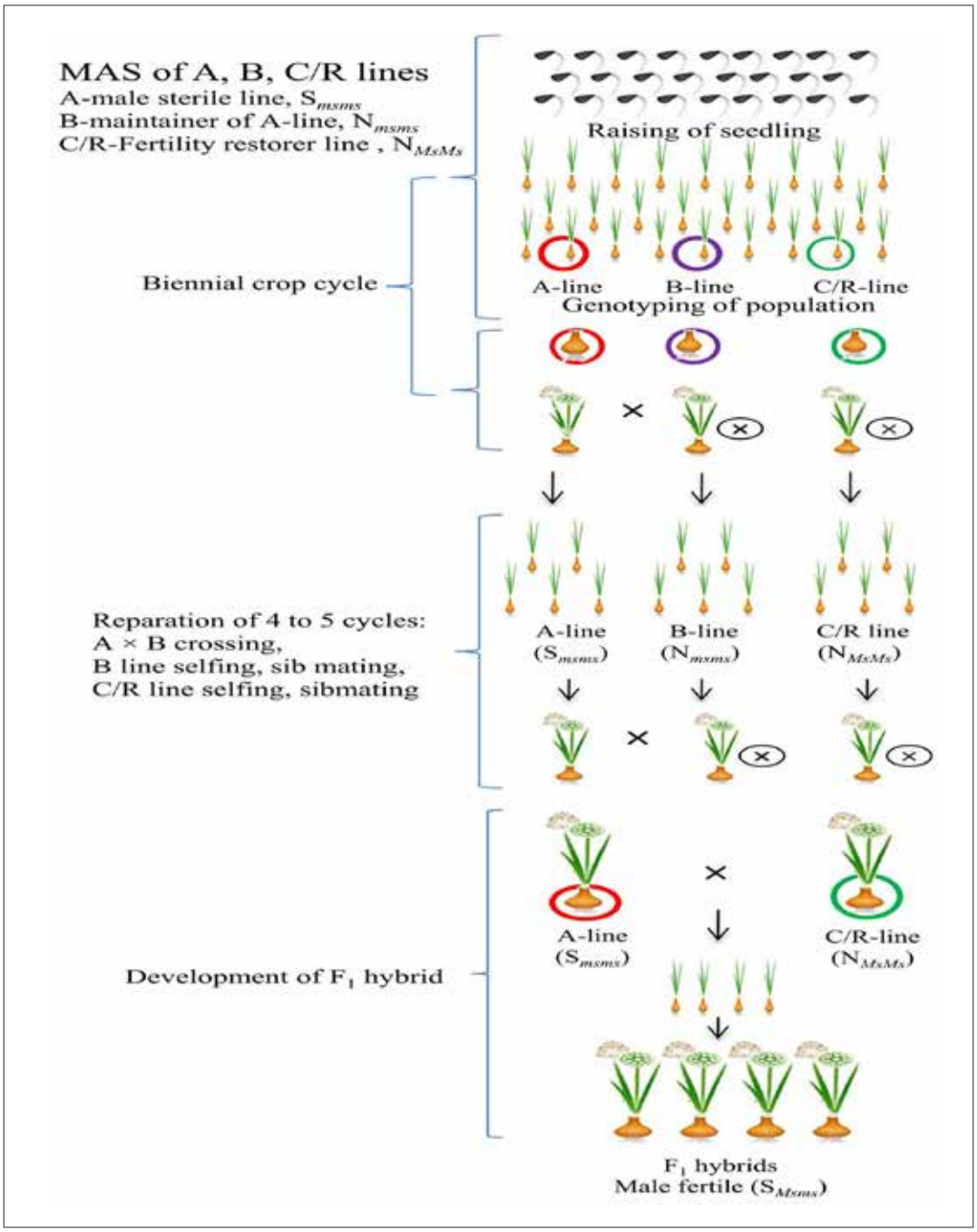

Figure 2. A schematic presentation of MAS based identification of three lines involved in hybrid seed production in onion (Manjunathagowda, 2021).

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