



Present status of haploidy research in onion (*Allium cepa*) – A review

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ABSTRACT

Research on haploid induction and their subsequent utilization in basic and genomic research and breeding is still at its infancy due to paucity of research efforts, difficulties in various critical steps, large genome size and limited publications. Haploid development in onion (*Allium cepa* L.) is only feasible through *in-vitro* gynogenesis and is influenced by genotype, geographic origin, genetic constitution, physiological stage, growth conditions and cultural conditions. Pollination with irradiated pollen has been reported to induce haploids in onion. However, more successful methodology is through whole flower bud culture. After haploid induction, chromosome doubling, for doubled haploids (DHs) induction, is another limiting factor. Whole basal plant as an explant and amiprofos-methyl as a chromosome doubling agent has been found to be safe and effective. Potential of DH lines has been explored through the study of hybrids using DH as parents and hybrids have been found uniform and promising compared to conventionally developed hybrids. DH line 'Onion haploid OH-1' has been released by USDA to serve as a responsive control for the extraction of gynogenic haploids. DH's have been successfully utilized in onion genomic research for identification of gene and genomic regions affecting quality traits like colour, restorer of male sterility (*Ms*) locus, bolting, fructan etc. Future studies need to focus on identification of haploidy inducing gene/QTLs, gynogenic responsive genes, establishment of efficient chromosome doubling techniques, restoration of fecundity of DH genotypes and methodology standardisation to transfer them in less/non-responsive desirable genotypes.

Key words: Doubled haploids, Genomics, Gynogenesis, Onion

Plants carrying gametophytic chromosome number in their sporophytes are termed as haploids. Haploid development is a tool for developing homozygous line (s) in time and resource efficient manner. This is useful in numerous plant breeding research experiments to develop varieties/hybrids apart from their use in genetic and biotechnological research (Szczyt 1995). Spontaneous development of haploid plants was first attempted by Bergner in *Datura stramonium* and reported by Blakeslee *et al.* (1922) followed by reports in tobacco, wheat and several other species (Forster *et al.* 2007). This technology has now been applied to over 250 plant species (Maluszynski *et al.* 2003).

Pollination with pollen of the same species (maize), irradiated pollen (cucumber, melon, squash, watermelon, apple, mandarin, blackberry, European plum, sweet cherry, kiwifruit, pear, carnation, rose, petunia, sunflower and *Nicotiana*), pollen from a wild relative (barley, potato) or unrelated species (wheat) has been reported to induce *in-situ* maternal haploids. Induction of *in-vitro* gynogenesis using un-pollinated flower parts has been found successful in wheat, barley, cucumber, squash, onion, sugar beet, gerbera

and sunflower (Murovec and Bohanec 2012). Androgenesis is the process of haploid regeneration through anther or microspore culture, whereas gynogenesis refers to the use of unpollinated female gametophyte for haploid development. The utilisation of gynogenesis/androgenesis for haploid and subsequent doubled haploid regeneration depends on crop type (recalcitrance towards androgenesis), its response (abnormal plant development like albino, rootless seedling etc) and pollination control mechanism acting on it like monoecy/dioecy, male sterility etc (Thomas *et al.* 2000, Bhat and Murthy 2007).

Haploidy in onion and subsequent diploidisation and their utilisation in breeding and genomic research holds great promise as it is very difficult to produce homozygous inbreds through selfing because of their biennial generation time, residual heterozygosity (King *et al.* 1998) and high inbreeding depression (Villanueva-Mosqueda and Havey 2001). Despite its significance, as one of the most important vegetable cum spice crop worldwide, genomic research in onion is still in infancy. This is partly due to its biological nature and enormous genome size (16.3 GB per 1C Nucleus) which is characterised by high frequency of duplication of recessive lethal alleles that remain masked in heterozygous state and maintenance of bulb population by open pollination (Havey 1993). Doubled haploid (DH) lines can be used as a tool to answer fundamental biological questions in onion

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as deleterious genes present in heterozygous population are fully expressed in DH lines. The strong selection pressure present in *in-vitro* regeneration stage eliminates genotypes with major phenotypic aberrations and enables the formation of vigorous and homozygous DH plants (Bohanec *et al.* 2002). Haploid technology as a tool to develop instant homozygous lines got a renewed attention by different plant research group in the last few years.

Haploid induction in onion through gynogenesis has been practiced for over 25 years (Jakše *et al.* 2010). Although DH technology deployment leads to the development of quicker and more economical homozygous onion lines than conventional breeding procedures (Bohanec 2002, Alan *et al.* 2003, 2004), yet research on onion genetic improvement in general and DH technology in particular is being carried out only in a few places. At present, research on DH development in onion has been reported from USA (Alan *et al.* 2007, Walker *et al.* 2006, Hyde *et al.* 2012, Duangjit *et al.* 2013), Turkey (Alan *et al.* 2014), India (Sivalingam *et al.* 2014, Mathapati *et al.* 2018, Khar *et al.* 2018) and Spain (Fayos *et al.* 2015). In this review, we have summarised the work on haploid breeding in onion carried out so far on various aspects which we hope will enrich the onion researchers and motivate new researchers to work on this fascinating technology for inbred development in onion and other *Allium* spp. for breeding purpose and identification of new genes.

Methods of haploid induction in onion

Pollination with irradiated pollens: Dore and Marie (1993) successfully attempted haploid development and subsequent gynogenic doubled haploid development through pollen irradiation by a Co₆₀ source using 150 Gy. The complexities associated with the protocol restricted its further utilisation by others and research on haploid induction through *in-vitro* gynogenesis assumed significance.

Gynogenesis

Anther culture, the most successful method of haploid development in other crops, has not been successful for onion (Keller and Korzun 1996). Campion (1984) reported nuclear cleavage in onion anther culture while Keller (1990) observed no androgenic development in 98027 anthers cultured in 25 different media. Culture of unfertilized ovaries and subsequent production of haploid plants through gynogenesis was first described in barley (San Noeum 1976) whereas, in onion, haploid development through gynogenesis was first reported by Campion and Azzimonti (1988) followed by Muren (1989) and subsequently by Campion and Alloni (1990). To date, only gynogenesis has been successfully utilised for development of DHs in onion.

Factors affecting haploidy/double haploidy in onion

Main factors that govern haploid induction and subsequent regeneration in onion are genotype of donor plant stage of flower/ovule development pre-treatment, culture medium and, cultural conditions. Different steps involved in development of DH onions are summarised in Fig 1.

Genotype of donor plant: Muren (1989), one of the pioneer researchers for onion haploid induction, classified genotypes into high, medium and low response groups based on their haploid induction efficiency and reported similar response of short day materials and long day materials. Genotype and type of explants are the main determining factors for gynogenesis. Genotypic differences for haploid induction based on day length, geographic origin and genetic constitution have been reported (Smith *et al.* 1991, Bohanec *et al.* 1995, Campion *et al.* 1995, Geoffriau *et al.* 1997, Javornik *et al.* 1998, Bohanec *et al.* 1999, Martinez *et al.* 2000, Alan *et al.* 2004). Jakše *et al.* (2010) used individual plants from the inbred long day onion lines, populations and hybrids and reported gynogenic efficiency in the range of 0.03 – 0.82% while the responsiveness of the selfed DH lines ranged from 0.00 – 0.63%. In India, Sivalingam *et al.* (2014) reported gynogenic efficiency in short day onion with a range of 0.9% (Bhima Shweta) to 4.5% (Bhima Shubhra) whereas Khar *et al.* (2018) observed that open pollinated varieties and hybrids were more responsive towards embryo induction followed by exotic and landraces. Fayos *et al.* (2015) reported variation in gynogenic responsiveness in Spanish onion germplasm and found that Valencia type commercial variety ‘Recas’ had the highest percentage (2.09%) of embryogenesis and the sweet cultivar ‘Fuentes de Ebro’ had the lowest percentage (0.53%). The differential response of genotypes with different origins as reported by various authors has been summarized in Table 1.

Stage of flower/ovule development: Developmental stage of explant is one of the crucial factors determining the success of gynogenic haploid induction (Yang and Zhou 1982, 1990, Muren 1989, Bohanec 1994, Mukhambetzhonov 1997, Bhojwani and Thomas 2001). The developmental stage of embryo sac or ovule, which plays a crucial role for reprogramming the pathway from gametophytic to sporophytic stage, is one of the most important factors in gynogenesis. In onion, haploid plants have been produced from ovules, ovaries or whole flower buds (Muren 1989, Campion and Alloni 1990, Keller 1990, Campion *et al.* 1992, Bohanec *et al.* 1995, Geoffriau *et al.* 1997, Michalik *et al.* 2000). However, ovule culture has been found to be the least efficient and the most time-consuming procedure. Flower bud or ovary culture exhibits similar induction frequencies concerning embryo induction (Campion and Schiavi 1994) and found to be less laborious (Bohanec *et al.* 1995, Bohanec and Jakše 1999). However, *in-vitro* culture of whole flowers, can also result in somatic regeneration, which needs to be distinguished from haploid embryos. Determination of embryo sac (ES) developmental stage is more difficult and time consuming. Therefore, most of gynogenesis experiments have focussed on empirical observation of embryo emergence after an appropriate time of *in vitro* cultivation. Muren *et al.* (1989) reported that ovaries excised 3-5 days before anthesis were more responsive, whereas Musial *et al.* (2001) suggested that embryo sacs at early stages of development which are capable of more parthenogenesis, might be more suitable



Fig 1 Steps in DH onion production. a) Onion umbel with 25-30% flower buds open, b) Selection of flower bud (3.5-4.5 mm) for inoculation, c) Flower buds after 1.5 months of inoculation, d) Induction of *in-vitro* plantlet directly through flower bud, e) Shifting the *in-vitro* induced plant to normal B5 medium, f) Cytological determination of haploid ($n=8$) status g) Flow cytometry for rapid identification of haploids, h) Subculturing for multiple shoot induction and root formation, i) Colchicine treatment under field conditions for DH induction, j) Hardening of plants under field conditions, k) Transfer of plants under field conditions, l) Growing under field conditions, m) DH onions harvested, n) Flowering of haploid, (H) and DH onion bulbs, o) Flower buds of haploid, p) Flower buds of DH onion bulbs.

for haploid induction than mature megagametophytes. The most ideal stage to culture the flowers is between 3 to 5 days before anthesis, which corresponds to the flower-bud size of 3-5 mm long (Alan *et al.* 2004). Musial *et al.* (2005) studied the development of onion embryo sacs *in-vitro* and gynogenesis induction in relation to flower size. They used two genotypes (inbreds) of highly responsive line B2923B and flowers of different size group, viz. small flower bud (2.3-3.0 mm), medium flower bud (3.1-3.7 mm) and large flower bud (3.8-4.4 mm). Gynogenic embryo was observed 14 days after culture when only mature ES were present in ovules. They further reported that the crucial period

for gynogenesis in onion is between 2 and 3 weeks of the culture when non-degenerated ES containing egg apparatus are available. Medium and large flower buds gave better response than the small flower buds. Fayos *et al.* (2015) opined that flowers of 3.5-4.5 mm in length are excellent material for doubled haploid production.

Pre-treatment: Variation due to external factors is eliminated through pre-treatment of stock plants to maximise gynogenic response in cultured flower buds. Stress treatments induce a shift from gametophytic phase to sporophytic phase and physical treatments like alteration of temperature (low/high), photoperiod (prolonged dark

Table 1 Differential response of onion genotypes with different daylengths and geographic regions towards *in-vitro* gynogenesis

Genotype	Day length	Geographic origin	Genetic structure	Regeneration rate (%)	Reference
IG 31	Long day	Northern Europe	Inbred	4.40	Geoffriau <i>et al.</i> (1997)
CG 11, CG 12		Northern Europe	Clone	0.70-0.13	
IG 34		USA	Inbred	2.30	
PG 48, PG 49, PG 47, PG 41		Eastern Europe	Population	0.96-0.17	
PG 44		Southern Europe	Population	0.13	
MSU5718B, MSU826B, MSU6111B, MSU8155B, B7728B, B2355B, MSU2399B, MSU2935B, BO223B, B2923B	Long day	USA	Inbred	19.3-0.2	Bohanec and Jakše (1999)
HRIGRU01, 007991, XPH3371, NVRS01, 005946			F1s	22.6-11.4	
AC43, B0223B, B1717A, B1828A		USA	Inbred	32.9-0.4	Bohanec and Jakše (2003)
B1828B, B2117B, B2133B, B2371C, B2923B, BYG15-23, MSU2399B, MSU2935B, MSU611-1B, MSU826B, W101B					
W205B, W404B, W52B					
NY 15-41-49	Long day	Cornell, USA	Inbred	4.1	Alan <i>et al.</i> (2004)
YIX Stock C, YIX Stock I, YIX Stock A, YIX Stock E		Cornell, USA	Open-pollinated	12.1-0.3	
Kasmer Red (99-511)		Home grown, Turkey	Open-pollinated	1.9	
Kasmer Red (99-1207)		Home grown, Turkey	Open-pollinated	0.6	
Southport Red Globe		Heirloom	Open-pollinated	1.7	
Santana, Tamara		Bejo Seeds, Oceano, California	Hybrid	11.9-1.6	
99-1165, 0-499, 0-501		Cornell, USA	Open-pollinated-synthetic	2.7-1.2	
Valcatore INTA, Cobriza INTA, Navidena INTA		Argentina	Open-pollinated	4.76-0.83	
B 0223 B, B 2923 B, MSU 2923 B		Ljubljana, Slovenia	Inbred	0.82-0.19	Jakše <i>et al.</i> (2010)
PI 233189, Timor 135			Population	0.10-0.03	
B1717B × B2923B			Hybrid	0.22±0.091	
Agrifound Rose, Agrifound Dark Red, Arka Kalyan, B780, Bhima Kiran, Bhima Red, Bhima Shakti, Bhima Shubhra, Bhima Shweta, Bhima Super, N-2-4-1, Pusa White Round, RLKM-1, W514	Short Day	India	Open-pollinated	3.5-0.9	Sivalingam <i>et al.</i> (2014)

period) or nutrient supply (starvation medium/ altered growth regulators) applied to donor plants or in *in vitro* culture may have a robust influence on embryo induction. Pre-treatment can be applied to different stages, such as intact flowers, isolated ovules or inflorescence with different duration. Puddephat *et al.* (1999) observed a 10-fold increase in gynogenesis when flower buds were cultured from stock plants maintained at 15°C compared to those

maintained at 10°C or ambient temperature of glasshouse. They obtained 49 embryos from 2,660 cultured flower buds and 45% of plantlets were successfully acclimatised to glasshouse conditions of which 68% were haploid. Alan *et al.* (2004) reported that flower buds (3-5mm) from stalks of plants stored at 10°C remained responsive to induction of gynogenesis and were comparable to fresh, large flower buds.

Culture medium: Gynogenic development of the

explant (flower bud) is greatly affected by the culture media formulations and the most often modified components are: source of organic nitrogen, carbohydrates, growth regulators since different stages of gynogenesis have distinct nutritional requirements, polyamines and gelling agent. During induction, ovaries require low levels of growth regulators and are to be kept in the dark or light, while for regeneration they are transferred to a medium with higher growth regulator concentration and incubated in light. Improvement in the haploid production efficiency revealed that there was close association between the explant type (ovary or flower) and the kind of auxin in the medium. Naphthalene acetic acid (NAA) was superior for ovary culture while 2,4-dichlorophenoxy acetic acid (2,4-D) gave good results in both ovary and flower culture (Campion *et al.* 1992). The requirement of media composition is affected by genotypes and Michalik (2000) reported the highest yield of gynogenic embryos on the B5 or BDS media supplemented with 2.0 mg/l 2,4-D and 2.0 mg/l 6-benzylaminopurine (BAP) followed by culturing in R regeneration medium (BDS supplemented with 1.0 mg/l NAA and 2.0 mg/l N6-2-isopentenyladenine). Earlier the basal media B5 (Gamborg *et al.* 1968), MS (Murashige and Skoog, 1962) and BDS (Dunstan and Short, 1977) were used (Muren, 1989, Campion and Alloni 1990, Keller 1990, Campion *et al.* 1992). Geoffriau *et al.* (1997) used B5 medium for induction and MS medium for regeneration. Flower bud culture protocols for induction of gynogenic haploids were established subsequently with some alterations of culture media including synthetic growth hormones (Bohanec *et al.* 1995, Martinez *et al.* 2000, Michalik *et al.* 2000). For gynogenesis induction in onion, earlier a two step protocol was followed which involved pre-culture of the flower buds followed by ovary or ovule isolation and culture in appropriate culture media. Later a simplified one-step protocol, consisting of culturing the whole flower bud in an induction medium until embryo stage was developed (Bohanec and Jakše, 1999, Jakše and Bohanec, 2003) was used and is still being followed. Medium composition is one of the crucial factors affecting gynogenesis. High sucrose concentration (Muren 1989) and supplements of 2,4-D and BA have been reported to enhance gynogenic responses (Campion *et al.* 1992, Bohanec and Jakše 1999). Other growth regulators like polyamines have been reported to be essential for growth and development of living tissues. Over the years, there have been modifications on culture media composition for improvement of gynogenic responses and it has been summarised in Table 2.

Effect of polyamines: Polyamines are a class of plant growth regulators widely distributed in various plant parts and abundant in flowers. An increase in polyamine synthesis has been reported to precede or accompany callogenesis (Ponchet *et al.* 1982), organogenesis (Aribaud *et al.* 1994) and somatic embryogenesis (Liu *et al.* 1997). The role of polyamines for induction of gynogenic embryo and regeneration of plantlets was firstly reported by Martinez *et al.* (2000) followed by Geoffriau *et al.* (2006), Ponce

et al. (2006), Ebrahimi and Zamani (2009) and Forodi *et al.* (2009). Martinez *et al.* (2000) reported that polyamine treatment reduced the embryo regeneration time (60-90 days) compared to previously reported time (46-152 days) and there was genotypic difference towards rate of haploid production and embryo generation capacity. The highest number of embryo was obtained with the treatment of 2 mM Putrescine and 0.1 mM spermidine, embryo maturation and plantlet formation was promoted by the application of 0.1 mM spermidine 15 days after culture. Geoffriau *et al.* (2006) reported that high level of free and conjugated spermidine and low putrescine + hydroxyputrescine/spermidine + spermine ratio at inoculation stage characterized the highest responsive varieties, high levels of spermidine and spermine at *in-vitro* culture stage characterized responsive varieties while high levels of putrescine and its derivatives at *in-vitro* culture stage characterized the lowest responsive varieties. Ponce *et al.* (2006) reported that cycocel (CCC) sprayed at the umbels increased the gynogenic embryo rate more than three times than the control. Ebrahimi and Zamani (2009) also supported the findings of Martinez *et al.* (2000) and reported the highest number of gynogenic embryos in Iranian cultivars with application of putrescine and spermidine. Forodi *et al.* (2009) also complemented the finding of other authors and observed that embryo production and regeneration of plantlets increased significantly with combined treatment of 0.5 mM spermidine along with 2,4-D and BA in the induction media.

Effect of gelling agent: Apart from the media composition, the gelling agent is another factor that may contribute to the success of the technique. Jakše *et al.* (1996) reported that gellam gum (Gelrite) doubled the number of regenerated embryos, compared to agar-solidified media, although the number of abnormal regenerants was higher with gellam gum. Ponce *et al.* (2006) achieved higher number of gynogenic embryos by using 7 g/dm³ gellan gum, and this number was not affected by the addition of putrescine to the media.

Chromosome doubling/Recovery of fecund DH lines

Chromosome doubling is the most important step, after obtaining haploids, to get sufficient number of DH lines of adequate fecundity. The success of this step depends on type of explants used and the antimitotic agent.

Type of explants for chromosome doubling: Three different types of explants have been used in chromosome doubling treatments: intact plantlet (Jakše *et al.* 2003, Grzebelus and Adamus 2004), split basal (Campion *et al.* 1995, Geoffriau *et al.* 1997), and whole basal explant (Alan *et al.* 2004). The intact plantlets are treated with antimitotic chemicals for 24-72 h in liquid or solid media. This method has restricted use due to difficulty in the differentiation of spontaneous or induced diploid plants high rate of mortality of the spontaneous diploid plants owing to cytotoxicity or polyploidization. In another method, the basal portion of young *in-vitro* gynogenic plants are sliced longitudinally into halves or quarters and the cut ends are exposed to

Table 2 Composition of various media formulations towards *in-vitro* gynogenesis

Type of media	Media	Hormones (mg/l)	Sucrose (g/l)	Reference
Induction media	B5	2,4-D (2) + BA (2)	7.5	Sivalingam <i>et al.</i> (2014)
Basal media	BDS	2,4-D (2) + BA (2)	100	Jakše <i>et al.</i> (2010)
Elongation medium	Half strength BDS		15	
Induction medium	Basal	2,4-D (2) + BA (2)	100	
Differentiation medium		TDZ (2)	100	
Induction media	BDS macro salts and B5 micro salts		100	Ebrahimi and Zamani (2009)
Expression medium	BDS macro and B5 micro salts		40	
Basal medium	BDS	2,4-D (2) + BA (2)	100	Ponce <i>et al.</i> (2006)
Induction medium	Modified BDS medium	2,4-D (2) + BA (2)	100	Musial <i>et al.</i> (2005)
Regeneration medium (R1)	Half-strength BDS		30	Grzebelus and Adamus (2004)
Regeneration medium (R2)	BDS		40	
Elongation medium	Half-strength BDS		30	Bohanec <i>et al.</i> (2003)
Induction media (A1)	B5 Media	2,4-D (2) + BA (2)	100	Michalik <i>et al.</i> (2000)
Induction media/Regeneration media (F6)	B5 Media		100	
Regeneration media (B)	MS Media	NAA (1) + 2iP (2)	100	
Regeneration media (R)	BDS Media	NAA (1) + 2iP (2)	100	
Induction medium	BDS macro, B5 microelements		100	Martinez <i>et al.</i> (2000)
Embryo culture medium	MS media		40	
Micropropagation medium	MS media	2,4-D (2) + BA (2)		
Induction medium (M1)	B5 Media	2,4-D (2) + BA (2)	100	Puddephat <i>et al.</i> (1999)
Induction medium (F6)	BDS Media	2,4-D (2) + BA (2)	100	
Induction medium (A2)	MS Media		30	
B1 media	MS Media	NAA (1) + 2iP (2)	100	
Propagation media (C1)	MS Media	IAA (1.5) + 2iP (2)	30	
Basal medium	BDS		100	Bohanec <i>et al.</i> (1999)
Embryo induction medium	BDS	2,4-D (2) + BA (2)	100	
Elongation medium			60	
Rooting medium		IBA (0.5)	40	
Induction medium	B5 basal medium	2,4-D (2) + BA (2)	75	Geoffriau <i>et al.</i> (1997),
Basal medium	MS Salts, Vitamins of Nitsch and Nitsch		30	Campion (1990)

anti-mitotic chemicals and subsequently cultured in suitable regeneration media. The unavailability of information regarding fecundity of DH lines produced through these two methods restricts their utilization for the exploitation of onion DH lines. In the third approach, whole basal explants from 2- to 4-month old *in-vitro* haploid plants are treated with antimutagenic chemicals for varying duration. Alan *et al.* (2004) reported the use of colchicine (200-400 mg/l) to the whole basal explants from 2- to 4-month-old *in-vitro* haploid plants and the plants became ready for transfer to the greenhouse in 1-3 months and following this method, they recovered fecund DH lines implying their possible use

in further research/breeding programme.

Types of antimutagenic agent for chromosome doubling:
In onion, the plants of reduced ploidy level are very stable and there is very low frequency of spontaneous diploidisation as compared to the other vegetable crops like *Brassica oleracea* (Bhatia *et al.* 2016, Bhatia *et al.* 2017, Bhatia *et al.* 2018). Jakše *et al.* (2003) obtained only one spontaneously doubled plant out of 107 haploid plants grown in the field for one season. Different antimutagenic agents like colchicine, dinitroaniline, oryzalin, trifluralin and phosphoric amide herbicide amiprofos-methyl (APM) at various concentrations and for various periods of exposure

are being used. Alan *et al.* (2004) reported successful application of colchicine 200-400 mg/l in liquid medium for 48 h for chromosome doubling. Grzebelus *et al.* (2004) reported that oryzalin, trifluralin and APM were better agents than colchicine due to their low toxicity as well as lower dose and APM was the optimal choice. Alan *et al.* (2007) suggested the use of combinations of three strategies, viz. use of antimetabolic agent (APM at 100-150 µM) on whole basal explants from *in-vitro* haploid plant, recovery of diploid plants from haploid plants via spontaneous chromosome doubling in somatic shoots regenerated from haploid flowers cultured *in-vitro* and use of a second cycle of gynogenesis to recover diploid plants from *in-vivo* tetraploid and mixoploid plant materials for maximum recovery of fecund DH plants in gynogenesis-derived populations for use in onion breeding programs. Recently, Fayos *et al.* (2015) recovered the highest number of doubled haploid plants through application of 25 µM APM in a solid medium for 24 h.

Determination of haploidy: Haploid induction through gynogenesis offers the possibility of using doubled haploid (DH) inbred lines in onion breeding. The ploidy status of the regenerated plants can be determined through root tip analysis (chromosome study), flow cytometry and marker analysis. Karyotype analysis for determination of ploidy of onion regenerants has been reported (Campion and Alloni 1990, Martinez *et al.* 2000, Ebrahimi and Zamani 2009) while flow cytometry (Geoffriau *et al.* 1997, Bohanec *et al.* 1999, Puddephat *et al.* 1999, Bohanec *et al.* 2003, Grzebelus and Adamus 2004, Alan *et al.* 2004, Ponce *et al.* 2006, Alan *et al.* 2007, Jakše *et al.* 2010, Fayos *et al.* 2015) has also been studied. Sivalingam *et al.* (2014) confirmed haploidy of gynogenically induced onion plants through SSR markers. Campion *et al.* (1995a) studied the homozygosity of gynogenic lines using morphological traits, esterase isozyme and RAPD markers and reported uniform morphological traits and no polymorphism of biochemical and molecular markers. Bohanec *et al.* (1995) reported gametoclonal variation in the cultured plants by the appearance of a novel RAPD band which was present in two out of 12 regenerants and not in the donor plants. Esterase isozyme analysis of diploid regenerants revealed 88.2% homozygosity of the regenerants (Bohanec *et al.* 1999).

Performance and application of DH lines

Genetics of haploidy: Bohanec *et al.* (2003) reported the genetics of gynogenic haploid production for the first time. The gynogenic efficiency is strongly affected by the genetic constitution of individual plant and they observed the highest frequencies of haploids in inbreds B0223B and B2923B. They selfed the lines, crossed these two lines with inbreds having low gynogenic ability, and testcrossed, and found that F₁ did not exhibit any superiority for gynogenic haploidy induction than the superior parent but significantly superior than the low producing parent. The self-pollinated progenies of B2923B-6 had higher frequency of haploid induction. They suggested that gynogenic haploid induction in onion has a genetic basis with significant non-additive

and environmental effect, it is quantitatively inherited with low production having dominance in B2923B. To increase gynogenic haploid production, S₁ family selection method has been suggested which would result in a significant response to the selection for haploid induction in onion populations.

Evaluation of DH lines for breeding program

The DH lines are completely homozygous and develop in a single generation whereas the traditionally bred inbreds are nearly homozygous with some residual heterozygosity which results in minor to major heterogeneity in the hybrids developed therein. Besides, the time, space and cost associated with onion inbred development are substantially higher than the doubled haploid production due to biennial generation time and high inbreeding depression owing to accumulation of recessive lethal alleles. Alan *et al.* (2004) reported production of morphologically uniform, vigorous fecund gynogenic lines through doubled haploidy and suggested that enhanced vigour of DH lines would help in maintenance of inbreds and production of uniform hybrids. Kim *et al.* (2007) compared doubled haploids and their hybrids with commercial cultivars of onion and reported hybrid vigour, higher uniformity and stability in the hybrids. They also echoed the similar view of Alan *et al.* (2004) regarding uniformity of doubled haploids and their potential as parents for uniform hybrid development. Hyde *et al.* (2012) reported the superiority of highly heterozygous material for DH development over traditional inbreds, which already suffers from inbreeding depression. They reported heterotic superiority for mean row weight and mean bulb weight of DH derived hybrids compared to their half-sib commercial hybrids. They also reported poor performance of DH derived hybrids, which may be due to the recessive or masking of the deleterious alleles. The DH lines are in demand in hybrid production of onion owing to less time requirement for inbred development which is cumbersome and lengthy process and higher inbreeding depression because of cross pollinated nature of onion. Higher uniformity, reduced space requirement production and maintenance cost as well as higher yield makes production of DH lines a viable option for hybrid development. Havey and Bohanec (2007) released 'Onion haploid OH-1' to be used as a responsive control for the extraction of gynogenic haploids of onion.

Application of DH lines in onion genomic research: CUDH 2150, a doubled haploid line from Cornell University (Alan *et al.* 2004) has been utilised for development of a linkage map to assign QTL for fructan content (*Frc*) and the genetic locus (*R*) conditioning red bulb colour (Baldwin *et al.* 2012) and genetic analysis of bolting (Baldwin *et al.* 2014). Duangjit *et al.* (2013) utilised two DHs, 5225 and OH1, for transcriptome sequencing to produce SNP-based genetic maps of onion using gynogenic haploids from the F₁ populations of these two DHs. They also used the same population and identified significant QTLs on chromosomes 1, 4 and 8 affecting anthocyanin concentration in onion

bulbs (Duangjit *et al.* 2014). Kim *et al.* (2004) used haploid population derived from H 6 to identify a new locus P, which is responsible for pink trait in onions. Same red male fertile doubled haploid line H 6 has been reported by various authors (Bang *et al.* 2011, 2013, Kim *et al.* 2015) to develop simple and efficient PCR markers linked to the restorer of fertility (*Ms* locus) gene. Abdelrahman *et al.* (2015) developed doubled haploid parents of onion, shallot and their F₁ hybrid to study the transcriptome and associated metabolome variability in order to identify genes that would help in abiotic stress protection towards development of stress-tolerant variety.

Future perspectives

The tangible impact of this technology in basic and applied research lies in development of highly efficient, consistent and genotype independent doubled haploid production system. The embryogenic potential of donor genotype, efficient diploidization technique and the capability for direct embryo to plant development are the three crucial factors affecting application of doubled haploid technology. Many onion genotypes across geographic locations, type (day length requirement) and genetic constitution (inbreds, synthetics, open pollinated) have been found less responsive to gynogenesis. The genetic basis for differential response of different genotypes is not yet known. There is a need to identify genomic regions and genes affecting gynogenic responsiveness in highly responsive genotypes. The mode of inheritance of desirable alleles involved in all crucial phases need to be studied thoroughly. Further the fate of DHs in different onion lines in terms of their morphological traits, uniformity, fertility and their superiority in development of onion hybrids versus the conventional methods needs to be worked out in detail in both long day and short day onions.

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