Production of doubled haploids in onion: A review

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ABSTRACT
Onion suffers from high inbreeding depression and, as a result, inbreds that are developed lack genotypic and phenotypic uniformity. Gynogenesis has emerged as a potential strategy to address this drawback. Efforts have been made since the 1980s for identifying highly-responsive genotypes and for overall improvement of the protocol for bettering gynogenic frequency in onion. Besides improving media composition, identification of responsive explants and increasing the chromosome efficiency has remained a major area of focus over the years. This article purports to review progress made thus far in the induction of gynogenic haploids in onion, and challenges/opportunities associated with it.

Key words: Onion, Allium cepa, gynogenesis, haploid, chromosome doubling

INTRODUCTION
Hybrid cultivars of onion are considered to be superior to open-pollinated varieties due to higher uniformity and expressed heterosis. Onion populations possess deleterious recessive alleles, and due to inbreeding depression, breeding lines can be selfed for only up to two or three generations in this biennial crop. Thus, with conventional breeding it is difficult to obtain homozygous inbreds for complete genetic and phenotypic uniformity in the resultant hybrid. Doubled haploid (DH) production is an alternative strategy for complete homozygosity and phenotypic uniformity to obtain inbred lines in onion (Bohanec, 2002). Spontaneous development of haploid plants was first described in Datura stramonium (Blakeslee et al, 1922), followed by tobacco, wheat and several other species (Forster et al, 2007). In situ induction of maternal haploids has been possible by pollinating 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). Induction of in vitro gynogenesis using un-pollinated flower parts has also been successful in several species like cucumber, squash, gerbera, sunflower, wheat, barley, onion and sugar beet (Murovec and Bohanec, 2012).

Haploidy has a great potential in onion breeding, as, hybrids are derived mainly from partial inbred lines. Partial inbred lines also require 14-16 years to be developed due to the biennial life cycle of the crop. Therefore, induction of di-haploids can greatly reduce the time and resources required for developing inbreds (Bohanec et al, 1995). The major factors affecting haploid induction include genotype, physiological condition of donor plants, developmental stage of the gametes (microspores, megaspore), pre-treatment, composition of culture medium, and physical factors during tissue culture (Murovec and Bohanec, 2012). Haploid plants may be obtained from male or female gametic cells. However, species differ in their ability to induce haploids via androgenesis or gynogenesis (Bohanec, 2002). It has been observed that response of anther to haploid induction is not successful in onion (Keller and Korzum, 1996). Haploid induction via un-pollinated flowers or ovaries is under practice for the last 20 years, after the first discovery by Muren (1989). A high rate of success in onion through gynogenesis was observed by Bohanec et al (1995), Luthar and Bohanec (1999) and Bohanec (2009). Induction of maternal haploids, called gynogenesis, can be achieved with
in vitro culture of various parts from un-pollinated flower such as ovules, placenta with ovules attached, ovaries, or whole flower-buds as discussed hereunder:

**Choice of explant, developmental stage and sterilization-protocol**

In onion, a large number of anther culture experiments failed to generate haploids (Keller and Korzun, 1996). However, gynogenic haploid induction could be achieved through culture of un-pollinated ovules/ovaries/whole flower-buds (Bohanec, 2002). Keller (1990) observed that ovule culture was the most laborious and yielded the lowest number of embryo regenerants. Therefore, this is no longer used for haploid induction in onion (Bohanec, 2002). Flower bud culture is the simplest way of inducing gynogenic haploids in onion and has been used in many recent studies by various workers. Bohanec (2002) and Bohanec and Jakse (1999) estimated that extraction of ovaries from pre-cultured flower buds vis-a-vis whole-flower culture required more labour, while, gynogenic response of ovary vis-a-vis flower culture was often similar. Also, whole-flower culture was found to have the disadvantage of growth of basal callus (not so in ovary cultures), resulting in production of low-quality haploid embryos. Flower culture has been reported to show a possibility of somatic regeneration from callus, though this was genotype-dependent. Currently, the most efficient method is to plate whole onion flowers, without sub-culture, to induce haploid plants from cells of the female gametophyte (Bohanec et al., 2003). According to Muren (1989), flower buds 3-5 days prior to anthesis were superior to either older or younger ones. Michalik et al. (2000) concluded that small young buds of 2.8-3mm length produced significantly fewer embryos than older ones of 3.5-4.5mm length, while displaying genotype specificity. Alan et al. (2004) collected unopened flowers of all sizes (2-5mm) and separated them into three size groups, viz., small (<2-2.5mm), medium (2.25-4.5mm) and large (>4.5mm) before culturing them in plates. Small flower buds responded poorly, whereas medium-sized buds gave the best results. Musial et al. (2001) reported that in onion, small and large flowers containing megaspore mother cells and mature embryo sacs, respectively, were less responsive than medium-sized flowers having 2-4 nucleate embryo sacs.

Before placing in culture, flowers were surface-sterilized with 96% ethanol for 1 min, followed by treatment with 10% sodium hypochlorite (60g/dm³ active chlorine) containing a few drops of Tween 20 for 15 min, and then rinsed thrice in sterilized water (Ponce et al., 2006). Bohanec et al. (1995) and Jakse et al. (1996) reported dissecting the flowers, followed by sterilization for 10 min in 5% (v/v) solution of sodium hypochlorite with a few drops of Tween 20 added in. Rinsing in sterile water followed this, after which the largest unopened flowers were selected and inoculated. Geoffriau et al. (1997) reported washing of the excised umbel in 96% ethanol for 1 min, sterilization in 0.5% sodium hypochlorite solution for 15 min, and rinsing thrice in sterilized distilled water.

**Pre-treatment**

The principal benefits of optimizing pre-treatment for stock plants was to eliminate variation arising from external factors, thus maximizing gynogenic responsiveness in cultured flower-buds of onion across its flowering season. Pre-treating the stock plant has been shown to significantly affect frequency of gynogenic embryogenesis from whole flower-bud cultures in a range of onion genotypes (Puddephat, 1999). Stress treatment is the most common factor affecting embryogenesis, where cold or heat shock, or starvation treatments are commonly used. Without imposing stress, a change from gametophytic to the sporophytic phase is very difficult. Pre-treatments can be applied to different types of explants, with varying severity and duration, resulting in different regeneration efficiencies as well (Chen et al., 2010). Puddephat et al. (1999) observed that pre-conditioning of stock ovaries significantly influenced gynogenic embryogenesis. Also, high illumination was found to be beneficial in onion. Flower buds excised from stock plants maintained at 15ºC were ten times more responsive than those taken from plants raised under glasshouse conditions, or held at 10ºC. It has been established that lowering donor-plant growth temperature in the final phase/s of flower development improves the efficiency of gynogenesis in edible onion. Alan et al. (2004) also reported that flower buds (3-5mm) from stalks of plants stored at 10ºC remained responsive to induction of gynogenesis and were comparable to fresh, large flower-buds. Bohanec et al. (1995) reported the use of Parafilm for sealing petri dishes and exposed them to 16-hr light/8-hr dark photoperiod at an illumination of 78μE m⁻²s⁻¹ at 25±1ºC. Jakse et al. (1996) exposed sealed petri dishes to 16/8hr photoperiod at 23-25 ºC and illumination of 78 μmol m⁻²s⁻¹. Geoffriau et al. (1997) cultured flowers at 20ºC night and 22ºC day temperature under a 16h photoperiod of 100 μmol/s/m² photosynthetically active radiation- PAR (400-700 nm) supplied by fluorescent
tubes (58W). Bohanec and Jakse (1999) exposed Parafilm-sealed petri dishes to 16/8 hr photoperiod at 21-23°C and 80µmol m²s⁻¹ illumination.

**Media composition**

**Basal mineral composition:** The three most-often used combinations of macro- and micro-elements have been reported as B₃ (Gamborg et al., 1968), BDS (Dunstan and Short, 1977) and MS (Murashige and Skoog, 1962). Chen et al. (2010) mentioned that organic-nitrogen source, carbohydrates and growth regulators are the most-often modified components. Generally, gynogenesis spans two or more stages, and each stage may have distinct nutritional requirements. During induction, ovaries require low levels of growth regulators and are then transferred to a medium with higher concentrations of growth regulators. Ponce et al. (2006) compared regeneration of gynogenic embryos at gellan-gum concentrations of 7g/dm³ and 3g/dm³, and the higher concentration was found to be more effective. Bohanec et al. (1995) tested a 2-step culture procedure for generating gynogenic plants using flower pre-culture, followed by ovule or ovary culture method of Campion and Alloni (1990). Further, Bohanec and Jakse (1999) found a single-step culture less time-consuming and three times more efficient than the two-step ovary culture. Bohanec et al. (1995) tested a 2-step culture procedure for generating gynogenic plants using flower pre-culture, followed by ovule or ovary culture method of Campion and Alloni (1990). Further, Bohanec and Jakse (1999) found a single-step culture less time-consuming and three times more efficient than the two-step ovary culture. Alan et al. (2004) compared the single-step culture (BDS medium) and two-step culture (BDS/B₁ medium) for induction of gynogenic plants, and concluded that, single-step strategy was more convenient for large-scale induction of haploids.

**Use of polyamines:** Polyamines have been reported as essential for growth and development of living tissues, by Ponce et al. (2006). Martinez et al. (2000) showed that putrescine and spermidine induced the onset of embryogenesis in onion and enhanced the number of gynogenic embryos / plantlets obtained. Embryo induction was greatest with a combined treatment of 2mM putrescine and 0.1mM spermidine. Addition of putrescine alone did not give any significant effect, whereas, use of spermidine after 15 days of culture promoted further embryo maturation and plantlet formation. Ebrahimi and Zamani (2009) reported production of highest number of gynogenic embryos in media supplemented with 0.01 mM BA, 0.01 mM 2,4-D, 2mM putrescine and 0.1mM spermidine in onion flower buds. Ponce et al. (2006) found putrescine to be inhibitory at high concentration 0.16g/dm³ in all the genotypes studied. CCC (2-chloroethyltrimethyl ammonium chloride) at a concentration 0.1 g/dm³ was found to increase gynogenic embryo production rate more than thrice when compared to Control. Genotype-specificity of medium influencing production rate of embryos was also observed. Various doses of growth regulators in different media combinations required for successful gynogenesis are tabulated below:

### Growth regulator/s used | Concentration in Induction Medium (mg/l) | Reference/s
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2,4-D + BAP | 2+2 | Muren, 1989; Campion et al., 1992; Bohanec et al., 1995; Jakse et al., 1996; Geoffriau et al., 1997
IBA + BAP | 2.03+1.25 | Keller, 1990
TIBA + ABA | 0.2+1 | Campion and Alloni, 1990
2,4-D + BAP; NAA + BAP | 2+0.12,1+0.12 | Campion et al., 1992
PAA + BAP | 100+2 | Jakse et al., 1996
2,4-D + BAP | 2+2 | Jakse et al., 2010; Bohanec and Jakse, 1999
2,4D + BA+ | 0.01mM | Ebrahimi and Zamani, 2009
Putrescine+ | 0.01mM |
Spermidine+ | 2mM+0.1mM |
BA+2,4-D | 2+2 | Puddephat, 1999

### Growth regulator/s used | Concentration in Regeneration Medium (mg/l) | Reference/s
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2iP+ IAA, 2iP+ NAA | 2+1.5, 2+1 | Puddephat, 1999
IBA | 1 | Muren, 1989
NAA+2,4D+ IAA+BAP+2iP | 1+0.4+1,5+2+2 | Campion and Alloni, 1990
TDZ | 2, 2 | Bohanec et al., 1995; Jakse et al., 1996
NAA + GA₃; NAA + BAP | 1+1,1+2 | Campion and Alloni, 1990
NAA+ 2iP | 1+2 | Campion and Alloni, 1990; Bohanec et al., 1995
IAA+BAP; | 1,5+2; 1,5+2; 1,5+1; 0,4+2; 2,4D+BAP; 2,4D | Campion and Alloni, 1990
+2iP; NAA+2iP+ ABA;NAA+ BAP+GA; BAP+GA, +TIBA | 1+2;1+2;1+0.2 |
IBA+BAP+GA | 2+0,1,2+3,5 | Keller, 1990; Campion et al., 1992

**Effect of genotype**

Genetic make-up of donor onion plant and growth conditions play the most important roles to succeed at gynogenesis. Theoretically, for haploid induction in onion, a maximum of 600% frequency can be expected. However, in practice, yields are low (Bohanec et al., 2001). Geoffriau et al. (1997) tested variable genetic material from different regions across the world. They found that only two out of...
18 onion cultivars showed a high gynogenic potential. In a similar study conducted by Bohanec and Jakse (1999), accessions from Europe, Japan and North America were analyzed, and they found the highest yield in North American cultivars. Very high variability was found among cultivars, and even within inbred lines. Michalik et al (2000) reported a maximum of 10% embryo yield in a breeding line out of 11 Polish cultivars and 19 breeding lines studied. Bohanec and Jakse (1999) demonstrated that, crossing responsive with non-responsive onion lines, resulted in increased gynogenic ability in the hybrid progeny. Jakse et al (2010) proposed an integrated method, wherein, the final proportion of haploid donors that regenerated DH plantlets doubled at the least, and reached up to 80%. Geoffriaia et al (1997) reported that among genetic structures, inbreds regenerated significantly better than synthetics. Regenerants from inbreds were the most vigorous, whereas, synthetics were confirmed to be good donor material for quality embryos.

**Determination of ploidy level and homozygosity**

Different methods have been used for analyzing ploidy level. Chromosome count was performed on root tips (Muren, 1989; Campion and Alloni, 1990; Keller, 1990; Bohanec et al, 1995; Campion et al, 1995) or shoot tip cells (Campion et al, 1995). Bohanec et al (1995) performed chromosome analysis of root tips by staining in vitro grown hydrolyzed (70°C, 3 min) root tip cells with acetocarmine (arrested in metaphase with 45mg/ 100ml 8-hydroxyquinoline). Martinez et al (2000) and Ebrahimi and Zamani (2009) determined chromosome number in root tip cells obtained from plantlets after treatmtn with 0.1% colchicine for 3h, fixed in 3:1 ethanol: glacial acetic acid, digested in 1N HCl at 60°C for 8 min and squashing in 45% acetic acid. For microscopic inspection of the karyotype, root tips were stained with 1% haematoxilin or 2% acetocarmine. Flow cytometry using leaf tissue has been a predominant method (Cohat, 1994; Bohanec et al, 1995; Campion et al, 1995; Jakse et al, 1996; Geoffriaia et al, 1997; Javornick et al, 1998; Bohanec and Jakse, 1999; Alan, 2004). A protocol for flow cytometry was developed by Bohanec et al (1995) and the samples were analyzed on a FAC-Sort flow cytometer. Bohanec and Jakse (1999) used this method to analyze ploidy level of regenerants. To release the nuclei, leaves were chopped with a razor blade in 1 ml 0.1M citric acid containing 0.5% Tween 20, and the suspension was filtered through 50µm nylon gauze filter. A three-fold volume of dye solution containing 5.25µg/ ml 4,6-diamidino-2-phenylindole in 0.4M disodium hydrogen phosphate was added to the filtered suspension. Flow cytometry measurements were performed with Partec PAS-II flow cytometer equipped with a 100W high-pressure mercury lamp. Partec Ploidy Analyzer was also used for ploidy analysis (Anon., 2007).

In diploid species, spontaneous diplody are very useful in plant breeding, as, these are more stable than dihaploid generation through haploids treated with colchincines. However, homozygosity of the regenerants needs to be proved (Geoffriau et al, 1997). Jakse et al (1996) submitted regenerated plantlets to isozyme electrophoretic analysis to establish the frequency of homozygous / heterozygous origin. Bohanec et al (1995) used a modified electrophoresis technique, where, approximately 200mg fresh weight of leaf tissue (young leaves sprouted from donor plant bulbs, or, in vitro grown leaves) was crushed in 200µl of extraction buffer consisting of 15% (w/v) sucrose, 50mM Tris-HCl (pH 7.1) in 0.5% (v/v) Triton X-100. The extract was then centrifuged at 26500x g for 5 min at 4°C; the supernatant was immediately loaded onto the gel. Stacking gel (2cm) consisted of 24.6 g/l acrylamide and 6.15g/l bisacrylamide, 4.0g/l Triton X-100, 0.7g/l ammonium persulphate, 0.6ml/l TEMED in 0.07M Tris (pH 7.8); and, the resolving gel consisted of 57.8g/l acrylamide and 2.2g/l bisacrylamide, 0.37g/l ammonium persulphate, 0.37ml/l TEMED, 2.0G/L Triton X-100 in 0.07M Tris (pH 7.8). As electrode buffer, 1g/l Tris and 5.52 g/l barbitone (pH 7.3) was used. Electrophoresis was carried out at 10°C for 3h at a constant voltage of 225 V. This method was used again by Bohanec and Jakse (1999), who had used another method of RAPD analysis earlier (Bohanec et al, 1995).

**Genome-doubling procedures**

The spontaneous doubling of gynogenic plants is a rare event in the bulb-onion (Bohanec, 2002). The major problem in genome doubling in onion is inaccessibility of the apical meristem of adult field-grown plants. Therefore, chromosome doubling of haploid onion plantlets should be attempted during in vitro propagation. Jakse et al (2003) mentioned that an efficient method for chromosome doubling should take into account survival rate and chromosome-doubling efficiency. Alan et al (2004) studied various parameters in chromosome doubling experiments with 100-400mg/l dose of colchicine, in liquid and solid media, for 24 and 48 hours. For high rate of recovery of diplody, exposure of basal explants from 2-4 month old in vitro haploid plants to 200-400 mg/l colchicine in liquid medium for 48h was
suggested, wherein, 10% survival of explants with APM (Amiprofos-methyl) treatment was reported. Geoffriau et al. (1997) compared the efficiency of colchicine and oryzalin, and the best results were obtained with either 2.5mM colchicine (up to 65.7% diploids) or 50µM oryzalin (up to 57.1% diploids). Both the chemicals induced mixoploids and affected plant regeneration, but better plant-quality was obtained with oryzalin. Grzebelus et al. (2004) reported oryzalin, trifluralin and APM as better agents than colchicine for in vitro chromosome doubling in onion tissue. However, APM is recommended due to its low toxicity. Bohanec and Jakse (1997) tested the effect of oryzalin and colchicine on halved basal shoots. Diploidization with oryzalin (67%) was better than with colchicine (21%). Jakse et al. (2003) reported that the 2-day treatment in liquid media supplemented with 50µM APM gave the highest percentage of diploids (36.7%), but the survival rate was reduced to 52.5% that in the non-treated control. Alan et al. (2007) compared the efficiencies of three antimitotic agents (APM, colchicine and oryzalin) and recommended APM (100 or 150µM) due to its low toxicity and ability to yield results comparable to that with colchicine (750 or 1000µM).

Thus, it can be concluded that complete homozygosity through DH approach can be attained in less time than traditional breeding approaches. However, genotype-specific protocols for induction of haploidy and chromosome doubling are required for success in onion.

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