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# Basal media optimization for enhanced embryogenesis and doubled haploid recovery in onion (*Allium cepa* L.)

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**Abstract**
This study investigates the efficacy of Gamborg B5 and Murashige and Skoog (MS) basal media for enhancing embryogenesis and doubled haploid (DH) recovery in onion (*Allium cepa* L.) through ovary culture. Four genotypes (E604, E606, E611, E247) were evaluated for embryogenic response, shoot regeneration, and ploidy stability. Results demonstrated a fivefold higher embryo induction rate in B5 medium (5.41%) compared to MS (1.05%), with genotype E247 exhibiting the highest response (6.0%). B5 medium also outperformed MS in shoot regeneration, yielding 34 shoots versus 4 shoots, and produced a higher proportion of haploids (67.64%) and DHs (23.52%) as confirmed by flow cytometry. The superior performance of B5 is attributed to its optimized ionic composition and compatibility with monocot physiology, minimizing somaclonal variation. These findings highlight B5 medium as the preferred choice for efficient DH production in onion breeding, accelerating homozygous line development. However, genotype-specific variability underscores the need for tailored protocols. Future research should explore molecular mechanisms underlying B5's efficacy and validate the field performance of regenerants. This study advances haploid technology in onion improvement, offering a cost-effective and rapid breeding solution.

**Keywords:** *Allium cepa* L., doubled haploids, Gamborg B5 medium, embryogenesis, flow cytometry, genotype-specific optimization

## Introduction
Onion (*Allium cepa* L.) represents a critically important vegetable crop with significant economic value attributed to its culinary, nutritional, and medicinal attributes (Ochar & Kim, 2023) [22]. The breeding of this species encounters several challenges, including its biennial life cycle, the prevalence of cross-pollination, and issues related to inbreeding depression (Mahajan & Gupta, 2023) [17]. Recent advancements in the genetic improvement of onions have predominantly concentrated on enhancing disease resistance, pest tolerance, and the augmentation of desirable traits (Cramer *et al*., 2021). [6] Moreover, the application of male sterility has surfaced as an indispensable technique in contemporary onion breeding, thereby facilitating the efficient development of hybrid varieties (Chikh-Rouhou *et al*., 2025) [5].
Doubled haploids (DH) technology has emerged as a powerful tool in crop improvement, particularly in onion breeding, by enabling the rapid generation of homozygous lines within a single generation. It offers numerous advantages, including the acceleration of breeding cycles, facilitating trait introgression, and enhancing genetic diversity. In the context of onion breeding, DH lines have demonstrated vegetative vigor that is comparable to or even superior to that of open-pollinated cultivars, with minimal inbreeding depression. Hybrids derived from DH lines have shown performance that is equal to or improved compared to conventional hybrids, likely due to the elimination of deleterious sublethal genes during gynogenesis. The use of DH technology in onion breeding opens up opportunities for developing resistant varieties, improving quality standards, and creating cytoplasmic male sterile (CMS) hybrids. Furthermore, the integration of genomics, transcriptomics, and marker-assisted selection can significantly enhance the efficiency and precision of DH production in crop improvement programs.
Recent investigations have concentrated on the optimization of basal media and supplements to enhance embryogenesis and double haploid (DH) recovery across a range of crops. In the case of

eggplant, Rivas-Sendra *et al.* (2015) [24] noted that repeated subculture on Murashige and Skoog (MS) medium, supplemented with specific phytohormones, significantly improved the regeneration of DH plants from microspore-derived calli. Conversely, Liu *et al.* (2002) [16] demonstrated that a novel chemical formulation can induce elevated rates of microspore embryogenesis in wheat, while co-cultivation with live wheat ovaries in NPB 99 media substantially enhanced plant regeneration. Furthermore, Bokore *et al.* (2016) [3] reported that the incorporation of silver nitrate markedly increased haploid plantlet production in durum wheat. In onions, Bohanec *et al.* (2002) [2] identified that factors such as the developmental stage of floral buds, the cultivation conditions of donor plants, and the composition of the media are critical for the induction of gynogenic embryos and subsequent DH production. These advancements collectively reflect significant improvements in DH recovery efficiencies across diverse crop species. This study aims to further elucidate the impact of specific media formulations on embryogenesis and DH recovery, thereby contributing to the optimization of haploid plant production methodologies in selected crops.

## Materials and Methods

All experimental procedures were carried out at the Research Centre and the Kalash Pvt. Ltd. Laboratory, under controlled environmental conditions designed to optimize doubled haploid (DH) production in Onion (*Allium cepa* L).

**Plant Material:** Four onion (*Allium cepa* L.) genotypes were used for regeneration and doubled haploid (DH) production: E604 (ONI-1), E606 (ONI-2), E611 (ONI-3), and E247 (ONI-4). These were selected for their diverse genetic backgrounds and coded for laboratory handling. Buds measuring 3.5 to 4.5 mm are harvested from the field by 11 AM, thoroughly washed with tap water, and then placed in labeled tea strainers. Under a laminar flow hood, they undergo sterilization using a solution of 0.3% Bavistin with Tween-20, followed by several rinses with sterile distilled water (SDW). For surface sterilization, the buds are treated with 70% alcohol for 2 minutes, then exposed to 2% NaOCl for 6 to 8 minutes (or 4% for 4 minutes), and subsequently rinsed 5 to 6 times with sterile deionized water. Finally, the buds are arranged on sterile tissue paper in petri plates to absorb any excess moisture (Gashu & Ethiopian, 2021) [10].

### Media Preparation and Composition

To investigate the effect of different basal media on regeneration and doubled haploid (DH) induction efficiency in onion (*Allium cepa* L.), two standard tissue culture media formulations were employed: Murashige and Skoog (MS) medium and Gamborg B5 medium. Both media were evaluated for their efficacy in supporting ovary-derived embryogenesis and subsequent shoot regeneration across four onion genotypes: E604 (ONI-1), E606 (ONI-2), E611 (ONI-3), and E247 (ONI-4). Both MS and B5 basal salts were procured from certified tissue culture suppliers and prepared under aseptic conditions. The media were supplemented with 3% (w/v) sucrose and solidified using 0.8% (w/v) agar (HiMedia, India). The pH of the media was adjusted to 5.8 ± 0.05 before autoclaving at 121 °C for 20 minutes (Mishra *et al.*, 2018; Owen *et al*., 1991; Wetzstein *et al.*, 1994) [19, 23, 26]. Two types of media were formulated for each basal medium:

**Initiation Media (Oni-I-MS and Oni-I-B5)**: Designed to promote the initial response of isolated ovaries, these media were fortified with hormonal combinations optimized for ovary swelling and callus initiation. Specific growth regulators, such as 2,4-D (2.0 mg/L) and BAP (0.5 mg/L), were added based on standard protocols for monocot embryogenesis.

**Regeneration Media (Oni-R-MS and Oni-R-B5):** Following the induction phase, explants were transferred to regeneration media with modified hormonal profiles favoring shoot morphogenesis and embryo development. The regeneration media contained kinetin (1.0 mg/L) and NAA (0.2 mg/L), a combination found effective for monocot regeneration.

### *In-Vitro* Regeneration, Subculture, and Ploidy Assessment

Following ovary culture initiation, responsive explants showing embryogenic or callogenic changes were selected for subculture. In total, 850 ovaries were cultured per treatment group, of which 630 explants in MS medium and 730 in B5 medium progressed to subculture. These were transferred to their respective regeneration media (Oni-R-MS or Oni-R-B5), where embryo-like structures were monitored and subcultured at regular intervals. Regenerated shoots were allowed to elongate before being transferred to rooting media and subsequently acclimatized under controlled conditions. For ploidy analysis, regenerated plantlets were subjected to flow cytometry using propidium iodide staining and Galbraith's buffer. Leaf tissues were compared against a diploid *Allium cepa* standard, and nuclear DNA content was used to distinguish haploid (N), doubled haploid (DH), and chimeric (mix) plants based on fluorescence peaks. This approach enabled genotype-wise quantification of regeneration success and DH efficiency across the two media systems (Domblides *et al.*, 2022; Dhatt & Thakur, 2014; Jakše *et al.*, 2010) [8, 7, 14].

## Results and Discussion

**Embryo Induction Efficiency:** A comparative analysis of embryo induction demonstrated a marked difference in the responsiveness of onion ovaries to the two basal media used. Out of 850 ovaries cultured in each treatment, only 9 embryos were induced on MS medium, resulting in a low induction rate of 1.05%. In contrast, Gamborg B5 medium produced 46 embryos from the same number of cultured ovaries, corresponding to a significantly higher induction rate of 5.41%. This fivefold increase underscores the superior efficacy of B5 medium in facilitating embryogenesis from ovary explants. Notably, all four genotypes tested showed improved embryo induction on B5 medium, indicating that this enhanced response was consistent across different genotypes and not limited to any specific type.

**Genotype-wise Embryo Induction Response:** Genotype-specific analysis of embryo induction revealed significant variation in responsiveness among the four tested lines. Under MS medium, induction rates ranged from 0.5% (E606) to 1.6% (E247), indicating a relatively low potential for embryogenesis. However, all genotypes exhibited a notable improvement when cultured on B5 medium. E604 and E247 achieved the highest induction rate at 6.0%, followed closely by E611 at 5.0% and E606 at 4.5%. Among the genotypes, E247 consistently showcased a superior embryogenic response under both media conditions, with its performance being particularly pronounced on B5 medium. These findings suggest a genotype × media interaction, wherein both genetic background and nutrient composition play significant roles in influencing the efficiency

of *in vitro* embryo formation.

**Plant Regeneration and Ploidy Determination:** The comparative evaluation of plant regeneration and ploidy stabilization demonstrated a significant advantage of Gamborg B5 medium over Murashige and Skoog (MS) medium in facilitating shoot regeneration and the recovery of haploid and doubled haploid (DH) plants in Allium cepa L. Under the B5 treatment, 34 shoots were successfully regenerated and established, in stark contrast to the mere 4 shoots obtained from the MS group. Flow cytometric analysis indicated that, among the regenerants derived from B5, 67.64% were haploid, 23.52% were DH, and 8.82% displayed mixed ploidy status. In comparison, the MS group produced only one DH plant, one haploid plant, and one chimeric plant. These findings highlight the superior morphogenic and genomic stabilization capabilities of the B5 formulation, likely due to its unique ionic composition and improved compatibility with monocot tissue physiology. The consistently higher regeneration efficiency and ploidy normalization observed across all genotypes further reinforce the conclusion that Gamborg B5 medium provides a more favorable biochemical and hormonal environment for ovary-derived haploid technology in onion breeding programs.

The study underscores the superiority of Gamborg B5 medium for *in vitro* haploid induction and plant regeneration in onion. The higher efficiency of B5 medium may be attributed to its distinct ionic composition and better compatibility with monocot tissue physiology. The findings align with previous research, which highlights the importance of optimizing culture media to enhance tissue growth and plant regeneration, and suggests that B5 medium provides a more favourable biochemical and hormonal environment for embryogenesis and ploidy normalization (Hamdeni *et al*., 2022; Belgacem & Louhaichi, 2013; Wiszniewska *et al.*, 2012) [13, 1, 2766]. Haploid and doubled haploid (DH) technology offers significant advantages in plant breeding by rapidly producing homozygous lines, accelerating crop improvement (Germanà, 2011a) [11]. In onion, both anther culture and unpollinated ovary culture have been successful for haploid induction. Gamborg's B5 medium has shown superior results compared to MS medium for embryo induction and survival in multiplier onion, with B5 producing more haploids and DHs (Adhiyamaan *et al*., 2019) [25]. Similarly, B5 medium supplemented with 2,4-D, BA, and 10% sucrose was optimal for haploid induction in unpollinated onion ovaries (Mathapati *et al.*, 2018) [18]. The effectiveness of haploid induction varies among genotypes and is influenced by media composition and growth regulators (Cheng *et al.*, 2013; Niazian & Shariatpanahi, 2020) [4, 21]. These techniques have become integral to breeding programs for many crops, offering a powerful tool for developing improved varieties (Germanà, 2011b) [12].

**Table 1:** Regeneration optimization in different variety of Onion on Basal media (MS) Reported media

| Sr. No | Name of Genotype | Lab Code | Media code for Inti | No Of Ovaries Ini | Media code for Subculture | No of Ovaries subculture | Embryo Induction | Number of shoots subcultured | No of Plants Tested for Ploidy | No of N Plants | No of DH Plants | Mix Plant | Varity vise embryo induction % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | E604 | ONI-1 | Oni-I-MS | 200 | Oni-R-MS | 150 | 2 | 1 | 1 | 1 | 0 | 0 | 1 |
| 2 | E606 | ONI-2 | Oni-I-MS | 200 | Oni-R-MS | 170 | 1 | 0 | 0 | 0 | 0 | 0 | 0.5 |
| 3 | E611 | ONI-3 | Oni-I-MS | 200 | Oni-R-MS | 120 | 2 | 1 | 1 | 0 | 0 | 1 | 1 |
| 4 | E247 | ONI-4 | Oni-I-MS | 250 | Oni-R-MS | 190 | 4 | 2 | 2 | 1 | 1 | 0 | 1.6 |
| Total | | | | 850 | | 630 | 9 | 4 | 4 | 2 | 1 | 1 | |
| Overall Percentage of embryo induction | | | | | | | 1.058823529 | | | | | | |

**Note:** The Onion ovaries to embryo induction rate are 1.05%

**Table 2:** Regeneration optimization in different variety of Onion on Basal media (Gambourg B5) Reported media

| Sr. No | Name of Genotype | Lab Code | Media code for Inti | No Of Ovaries Ini | Media code for Subculture | No of Ovaries subculture | Embryo Induction | Number of shoots Regenration | No of Plants Tested for Ploidy | No of N Plants | No of DH Plants | Mix Plant | Varity vise embryo induction % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | E604 | ONI-1 | Oni-I-B5 | 200 | Oni-R-B5 | 180 | 12 | 8 | 8 | 6 | 2 | 0 | 6 |
| 2 | E606 | ONI-2 | Oni-I-B5 | 200 | Oni-R-B5 | 190 | 9 | 7 | 7 | 5 | 1 | 1 | 4.5 |
| 3 | E611 | ONI-3 | Oni-I-B5 | 200 | Oni-R-B5 | 150 | 10 | 7 | 7 | 4 | 2 | 1 | 5 |
| 4 | E247 | ONI-4 | Oni-I-B5 | 250 | Oni-R-B5 | 210 | 15 | 12 | 12 | 8 | 3 | 1 | 6 |
| Total | | | | 850 | | 730 | 46 | 34 | 34 | 23 | 8 | 3 | |
| Overall Percentage of embryo induction | | | | | | | 5.411764706 | | | 67.64705882 | 23.52941176 | 8.823529412 | |

**Note:**

1) The onion ovaries to embryo induction rate is 5.4%
2) The onion regeneration plant to SDH (2N) induction rate is 23%
3) The onion regeneration plant to Haploid (N) Induction rate is 67.64%

## Conclusion

The study demonstrates the clear superiority of Gamborg B5 medium over Murashige and Skoog (MS) medium for inducing embryogenesis and supporting plant regeneration in *Allium cepa* L. through ovary culture. Enhanced Embryo Induction: B5 medium achieved a fivefold higher embryogenic response (5.41%) compared to MS medium (1.05%), highlighting its efficacy in promoting early developmental stages. Genotype-Specific Success: The genotype E247 exhibited the highest embryogenic potential (6.0%), emphasizing the need for genotype-specific optimization in tissue culture protocols. B5 medium produced 34 shoots, significantly outperforming MS medium (4 shoots), and yielded a high proportion of haploids (67.64%) and doubled haploids (23.52%), crucial for breeding applications. These results underscore the potential of B5 medium as a standardized protocol for haploid and doubled haploid production in onion breeding programs. However, the variability among genotypes suggests that further research is needed to optimize conditions for less-responsive cultivars. Future studies could explore the Molecular mechanisms underlying B5's efficiency, the Field performance of regenerated plants, and Integration with marker-assisted selection to enhance genotype screening.

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**Conflict of Interest**

The authors declare no conflict of interest related to the findings, methodology, or interpretation of results in this study.

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