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An Optimized Protocol for Seed Sterilization and Shoot Regeneration from Mature Embryo in Wheat (*Triticum Aestivum* L.) *var*. HD2967

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Abstract: In the present study, an efficient protocol for seed sterilization was developed. This protocol used a 1.0 % bavistin solution, which provides a protective response against fungal contamination. It was observed that there was no significant difference among the effective sterilization treatments in comparison to seed germination and seedling length under *in vitro* conditions. Furthermore, the embryo excised from bavistin-treated seeds showed improvement in callus induction and shot regeneration in *var*. HD2967. 0.1% $HgCl_2$-SDS solution used for seed sterilization was also optimized with 2.5 to 3.0 minutes as the best-suited duration, providing a completely sterilized environment for the seeds for healthy and fast growth. In addition, plant growth regulators (PGRs) were further used to screen the most effective concentration for callus to shoot formation using mature embryos. Auxin concentration of 2.0 mg/l in MS agar media was quite effective for callus induction from embryo explant. For shoot regeneration (SI-7) combination is suitable for a maximum number of shoots per callus. This combination of 2, 4-D, Zeatin, $CuSO_4$, and TDZ showed good regeneration in the wheat variety HD2967. The optimized protocol displayed 100% callus induction and up to 80-85% regeneration in HD2967, with a high reproducibility rate paving the way for regenerating plantlets from mature wheat embryos.

Keywords: *Triticum Aestivum var*. HD2967; mature embryos; regeneration; callus; shooting; rooting.

1. Introduction

An explant should be properly sterilized to regenerate the healthy and multiple plantlets to minimize contamination chances upon exposure. Using seeds as an explant could significantly impact the subsequent plant development as a massive array of viable microorganisms inhabits them. Since whole-wheat grain has been widely used, it is necessary to regenerate the plant using an advanced sterilizing protocol. The sterilization technique has been a primary step to eliminating contamination in the tissue culture approach. Further, surface sterilization is the key step in plant tissue culture to improve callus induction and regeneration efficiency. In the present study, we have established an efficient and less time-consuming protocol for seed and embryo sterilization. A combination of 70% ethanol, SDS-$HgCl_2$, and bavistin has been used to sterilize whole wheat seeds. The use of bavistin in the sterilization process creates a protective zone surrounding the seeds so that there is a negligible chance of fungal infection. Ethanol exposure needs to be standardized for seed sterilization as

it dehydrates the bacterial cells. Beyond the optimum range, ethanol could harm the plant cell as well.

Furthermore, SDS, an anionic surfactant, is known to remove dust particles from the surface, whereas $HgCl_2$ as a bleaching agent, which releases chlorine gas, leads to oxidative stress conditions in the bacteria cell [1]. The wheat seed has been one of the major food requirements consumed almost worldwide. However, to achieve the food requirements for the growing population as per the 2050 target of 140 million tons, it is essential to regenerate multiple plantlets from superior explants to enhance production. Plant tissue culture has been one of biotechnology's vital and fascinating branches, which could provide an enriched advantage over crop production and improvement programs [2- 4].

It is also reported that *in vitro* power of embryo regeneration can solve many practical breeding problems to facilitate plant breeders [5,6]. Moreover, this could also assist in delineating various nutritional aspects and metabolic, developmental, and biochemical pathways. Furthermore, the tissue culture technique helps induce numerous regenerable cells and plants aseptically *in vitro*, which are further helpful in evaluating various effects of plant hormones and biotic/abiotic environmental stress conditions using transgenic analysis [7,8]. Here we have chosen HD2967, one of the important Indian agronomical cultivated genotypes, which was released in the year 2011. HD2967 is a high-yielding variety used by the farmers of Haryana–Punjab and western UP in India on a large scale. The major advantage of the HD2967 variety is its capability to withstand a wider temperature range, resistance to yellow rust disease, and shorter length, which further prevents it from falling during strong winds. Callus formation was optimized using various auxin concentrations, i.e., Dichlorophenoxyacetic acid (2,4-D), using the mature embryo. An optimum combination of 2,4 D, zeatin, copper sulfate ($CuSO_4$), and TDZ (Thidiazuron) was used for multiple plantlets.

2. Materials and Methods

2.1. Collection of germplasm.

The seeds of a popular variety of wheat HD2967 that is frequently grown in the north zone were used in the present study. Seeds were maintained in the plant tissue culture laboratory of the Department of Biotechnology at M.M. (Deemed to be University), Mullana (Ambala) at 4ºC till further use.

2.2. Seed sterilization.

Approximately 250-300 seeds were initially put in a 100 ml beaker and rinsed under running tap water to remove the external contaminants. To avoid any bacterial contamination, seed sterilization was done under aseptic conditions. Further, the seeds were rinsed with distilled water followed by 1% bavistin. Bavistin is an antifungal powder that works systemically for the surface sterilization of seeds. After this, the seeds were thoroughly washed with sterile distilled water (4-5 times) to remove all the traces of bavistin from the seed surface. The sterilized seeds were kept in fresh water for embryo excision under aseptic conditions.

2.3. Excision of embryo and embryo sterilization.

The sterilized seeds were poured into a Petri dish, soaked under distilled water overnight at 4°C, and the embryo was excised using a sharp needle between the forefinger and

thumb. Embryos were kept in a 90 mm Petri plate and washed the embryo with autoclave-distilled water twice. They rinsed the embryos with 70% ethanol, followed by washing the embryo with distilled water and adding 0.1% SDS+$HgCl_2$, mixed vigorously clockwise and anticlockwise for 3.0 min, and washed the embryos 5 times with autoclaved distilled water to remove all the traces of $HgCl_2$.

2.4. Callus induction.

Stock solutions were prepared per the concentration given by Murashige and Skoog (MS) [9] with modification in PGR. Approx. 40–50 embryos with scutellum up-side were kept in each Petri plate for further callus induction with different concentrations of auxin (2,4-D) varying from 1.00 to 3.0 mg/L. The Petri plates were sealed with parafilm and incubated in the dark at 25 ± 1°C for the next 7 days.

2.5. Regeneration.

After 5 days, the callus was spreading outward and attained like a spongy tissue shape gradually with time. After 10 days, healthy callus was cut into several small pieces using a sterilized scalpel blade and kept on a regeneration medium containing 2-4 D, Zeatin, $CuSO_4$, and TDZ in different concentrations, as shown in Table 1. The incubation conditions for regeneration were relative humidity (RH) 60%, temperature 25 ± 1˚C, and 16/8 h photoperiod with 2000 lux white fluorescent light under tissue culture racks in the culture room. Multiple shots were separated under aseptic conditions before transfer into the rooting medium.

2.6. Rooting.

For rooting the regenerated plantlets, half the strength of MS medium was sufficient to grow roots. 50 ml of rooting medium without any PGRs were poured into each jam bottle and pushed slightly in the medium using long sterilized forceps. Roots were inducted from implanted shoots in the medium after 7 days. The incubation conditions for rooting were the same as maintained in a shooting.

2.7. Acclimatization.

Initially, uplift the cover of jam bottles for 3-4 days. In the next few days, plantlets will adapt to the environment. Plantlets were pulled from the media with forceps and washed with distilled water. The plantlets with media-free root networks growing in *in vitro* conditions were transferred to 4-inch pots containing soil mixed with peat moss and vermicomposting in a 1:1:1 ratio and covered with poly bags from the open side of bottles to maintain the humidity for new plantlets. Furthermore, 4 days later, small holes were created with the sharp edge of forceps in the poly begs so that plantlets could acclimatize themselves to the natural environment and grow for maturity.

3. Results and Discussion

3.1. Seed sterilization.

Seed sterilization is a crucial step in tissue culture, affecting seed viability and regeneration efficiency. Approximately 250 -300 seeds in each lot were kept in a 50 ml falcon

tube and washed under running tap water. Taking more than 300 seeds in one lot increases the chances of contamination. To avoid any surface contaminants, antifungal bavistin treatment (1%) was given to the seeds. Bavistin is an antifungal powder that works systemically for the surface sterilization of seeds.

Further, 0.1% $HgCl_2$-SDS solution was used for further sterilization. It was observed that the chances of contamination were more likely during the initial 2.5 minutes. As the exposure time increased, contamination was reduced (as shown in figure 1), with negligible contamination observed beyond 3.0 minutes. Therefore, the optimum sterilization duration for HD2967 lies between 2.5- 3.0 minutes. Further washing (5 times) with autoclaved distilled water removes all the traces of $HgCl_2$ froth from the falcon tube.

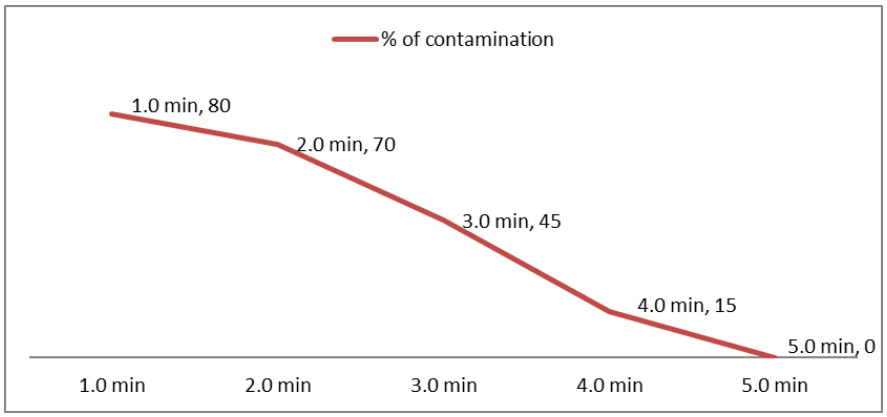

Figure 1. Percentage of seed contamination decreased with the increase of the incubation time of $HgCl_2$+SDS (0.1%).

3.2. Embryo excision and sterilization of excised embryos.

Sterilized seeds were kept under autoclaved water in a sterile Petri dish at 4°C overnight so that seeds were imbibed in water. The next morning, using a sharp needle, embryos were excised with careful hands in a laminar hood. The excised embryos were kept in a separate Petri dish in autoclaved distilled water to avoid dryness. If the embryos are dried, their protein content gradually decreases, having an adverse effect on callus formation. For embryo sterilization, excised embryos were rinsed twice with sterile distilled water, followed by rinsing with 70% ethanol for 25 sec and then washing once with sterile distilled water. Shake the embryos with 0.1% SDS-$HgCl_2$ for 2.0 min, followed by washing with sterile distilled water continuously till the froth as a result of SDS is completely removed under aseptic conditions.

3.3. Callus induction.

Sterilized embryos were kept on sterile tissue paper to absorb the extra water under aseptic conditions. The embryos were uniformly placed on MS medium containing 2,4 D at varying concentrations (0.2 mg/l, 0.5 mg/L, 1.0 mg/L, 1.5 mg/L, 2.0 mg/L, 2.5 mg/L and 3.0 mg/l). The positions of embryos are kept in such a way that the upside of the embryo should be slightly dipped in the medium for the ease of getting a nutrition-induced *mass* of undifferentiated *cell*s. The Petri plates having embryos were sealed with parafilm and kept in the dark for 7 days. Callus formation started within the next 3 days. Within 4-5 days, the callus appeared as a spongy tissue spread outwards. Among the different concentrations of 2,4 D, it was observed that 2,4 D was a suitable phytohormone for callus induction at 2.0 mg/l concentration. As the concentration of 2,4 D was increased above 2.0 mg/L, callus formation

was induced further, but the precocious germination rate was also increased. Thus, 2,4 D in 2.0 mg/L concentration is the best-suited medium for callus induction in HD2967, as more than 90% of mature embryos were transformed into a healthy callus.

Table 1. Shoot induction (SI) medium composition.

Shoot induction Medium	Composition of medium
SI-1	2,4 D (0.2mg/L)+CuSO4 (10 mg/L)+Zeatin (5.0 mg/L)+ TDZ (0.5 mg/L)
SI-2	2,4 D (0.2mg/L)+CuSO4 (12 mg/L)+Zeatin (5.0 mg/L)+ TDZ (0.5 mg/L)
SI-3	2,4 D (0.2mg/L)+CuSO4 (14 mg/L)+Zeatin (5.0 mg/L)+ TDZ (0.5 mg/L)
SI-4	2,4 D (0.2mg/L)+CuSO4 (16 mg/L)+Zeatin (5.0 mg/L)+ TDZ (0.5mg/L)
SI-5	2,4 D (0.2mg/L)+CuSO4 (17 mg/L)+Zeatin (5.0 mg/L)+ TDZ (0.5mg/L)
SI-6	2,4 D (0.2mg/L)+CuSO4 (17 mg/L)+Zeatin (5.0 mg/L)+ TDZ (0.75 mg/L)
SI-7	2,4 D (0.2mg/L)+CuSO4 (17 mg/L)+Zeatin (5.0 mg/L)+ TDZ (1.0mg/L)

3.4. Shoot induction from an undifferentiated mass of cells on the regeneration medium.

The undifferentiated mass of cells was whitish with a transparent appearance. These white brittle like structures were further kept on regeneration medium containing MS + 2, 4 D (0.2 mg/l) + $CuSO_4$ (17 mg/l), Zeatin (5.0 mg/l) and TDZ (1.0 mg/l), for 7 days. Bavistin (5 mg/l) was added to the medium to avoid fungal infection during shooting. However, the use of bavistin during shoot regeneration is optional. After 15 days of subculture, the emerging shoots were observed in jam bottles having the same medium. The shoots were kept on this medium for the next 15 days, and then each shoot was separated from a bunch of shoots regenerating in jam bottles under aseptic conditions with gentle handling. Further, transferred the shoots were into test tubes containing MS without PGRs for rooting.

3.5. Hardening and acclimatization.

Half the strength of MS medium is sufficient for further rooting from regenerated shoots. Within one week of incubation at room temperature under aseptic conditions, roots start emerging from the shoots. After 15 days, the seedlings were expelled from the medium and washed in distilled water to remove all traces of the medium. Now medium free seedlings were planted in a mixture of soil containing a 1:1:1 ratio of soil: peat moss: vermicomposting in 4 inches pots. To provide the humid environment, seedlings were covered with polybags. After 5-6 days, a small punch was created around the polybag to adapt the seedlings to the external environment. Furthermore, seedlings were adopted in natural conditions and grown to continue for further analysis.

Table 2. Representation of percentage of callus induction and shoot generation from mature embryos of HD2967.

	No. of seeds	% of Mature embryo	% of Callus formation	% of Regeneration
Lot 1	600	80-90 %	~95 %	~85 %
Lot 2	600	80-90%	~90 %	~80 %
Lot 3	600	80-90%	~90 %	~85 %
Lot 4	600	80-95%	~87 %	~80 %
Lot 5	600	80-90%	~92 %	~80 %

3.6. Discussions

This study focused on seed sterilization protocol and assessing plant growth regulators, which influence callus formation and shoot regeneration, to observe their optimal concentrations using mature embryos in wheat germplasm. Seed sterilization is the most vital step in plant tissue culture. With an efficient seed sterilizing method, there is no passage of

contamination in plant regeneration. The previous reports showed that long-term treatment of sterilizing agents such as sodium hypochlorite, calcium hypochlorite, mercuric chloride, and ethanol affect the callus induction and regeneration efficiency [10- 12]. In this sterilization protocol, bavistin-treated seeds showed less fungal infection than non-treated seeds. It was found that embryos excised from bavistin-treated seeds showed more than 90 % callus formation (Table 2), as bavistin is an antifungal powder that is more efficient in removing the seed surface and seed-borne diseases [13= 15].

Furthermore, Bavistin is very helpful in resolving fungal contamination during plant tissue culture, especially in callus formation and shoot regeneration protocols [16- 19]. This sterilizing protocol is comparatively cheaper, less time-consuming, and highly efficient for regeneration, especially in wheat crops. Thus, the optimized protocol could reduce wheat tissue culture's bacterial and fungal contamination risk.

An undifferentiated mass of cells has been used to generate multiple plantlets in various crop systems. The auxin 2, 4-dichlorophenoxy acetic acid (2, 4-D) itself, and in combination with another cytokinin(s), is commonly used to induce callus formation [10,11,20-23]. Here, we have used different concentrations of 2,4 D (0.5 to 3.0mg/l) for callus formation (Fig 2). It was observed that the percentage of callus induction was increased with increasing concentration of 2, 4-D from 0.5 mg/L to 2 mg/L. However, with the increasing concentration of 2, 4-D above 2.0 mg/L i.e., 2.5 mg/L and 3 mg/L, the processing of callus is faster, but the rate of precocious germination also increases. Hence, the optimum 2, 4 D concentration was found to be 2.0 mg/L for callus induction. It is observed that puffiness of callus formation occurred within one week of incubation on callus induction medium under dark conditions at room temperature. In addition to callus formation, 2,4 D has a strong resistance capability to enzymatic degradation and conjugation in plant cells [24- 26]. It is also reported that a high dose of 2, 4-D affects the cellular organization and shoot meristems in wheat genotypes [27-29].

In the current study, regeneration medium contained MS+ 2, 4-D (0.2 mg/L) + $CuSO_4$ (15.0 mg/L) + Zeatin (5.5 mg/L) and TDZ (2.0 mg/L) which showed optimal regeneration within one week (Fig.2). A low concentration of 2, 4D supports the regeneration in wheat via embryo callus. It is also known that it either suppressed the rooting or enhanced the shooting in a combination of other growth regulators [11,30]. In addition, heavy metals improve regeneration efficiency via plant tissue culture [31,32]. Cu, Ag, Co, Mn, Ni, and Zn at specified concentrations have been reported to stimulate morphogenesis [33- 35]. Cu^{+2} ions work as stress-inducing agents and are responsible for shoot promotion [36]. Here, we have standardized the optimum concentration of CuSO4 (17 mg/L) which produced maximum plantlets per callus with green and healthy leaves.

Similarly, with the increase in the concentration of $CuSO_4$ in the medium, the number of shoots per callus also increases, but at higher concentrations, plantlets start getting wrinkled with rosette leaves [3,28,37]. Due to the antimicrobial properties of Ag^+ ions, $AgNO_3$ is also used as a disinfectant in barley with broad-range biocidal activity to kill bacteria, fungi, viruses, and protozoa [38]. It is also known that N-phenyl-N'-1,2,3- thiadiazol-5-ylurea (thidiazuron, TDZ) can significantly improve the morphogenic response of explants for shoot induction [39]. It is a potent growth regulator in cereals [40]. TDZ alone and in combination with 2,4 D and NAA enhanced the shoot formation more than 3 fold compared to BAP and Kinetin [41- 43]. It is also reported that TDZ increased regeneration frequency significantly in nine popular Indian wheat varieties [11,44,45]. Similarly, in our study, the combination of 2, 4-D, Zeatin,

$CuSO_4$, and TDZ showed good regeneration in the wheat variety HD2967. However, mature embryos showed good callus induction and regeneration using the above-standardized protocols, which could be a good choice for other wheat varieties. Thus, optimized media displayed good and healthy callus induction and shot regeneration from mature embryos in the wheat variety HD2967 and could also be utilized for other germplasm.

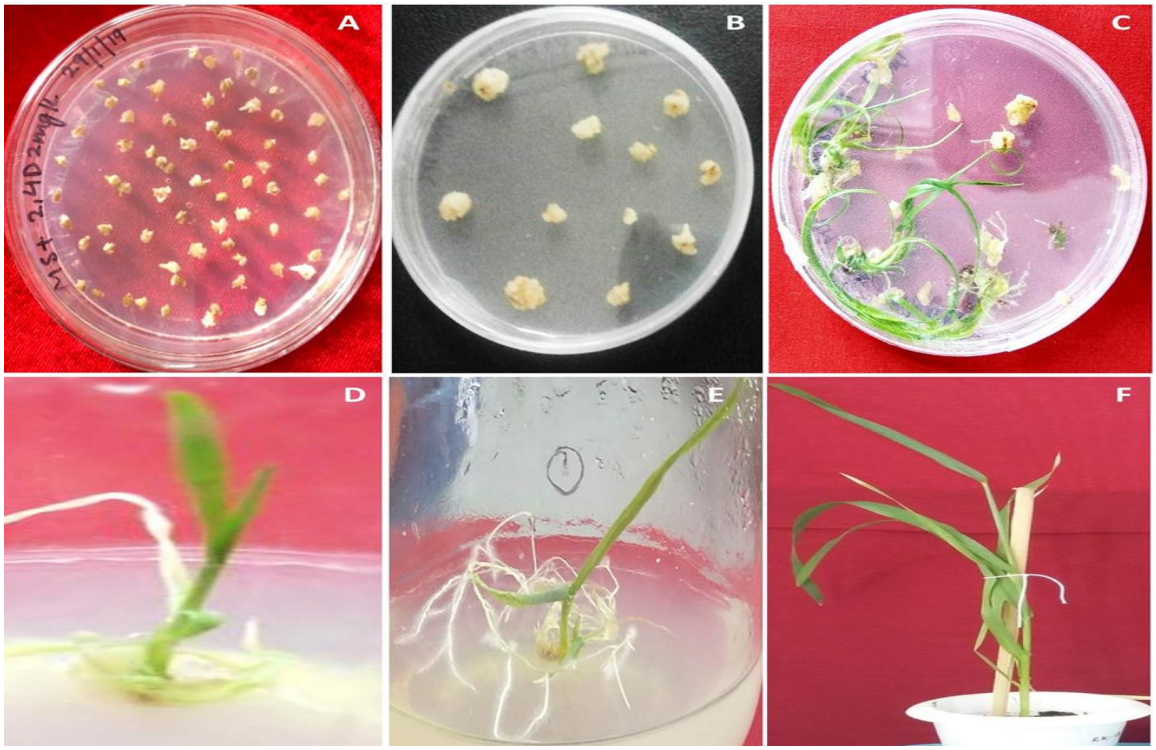

Figure 2. Methodology of regeneration of wheat plants from a mature embryo. (A) callus induction from a mature embryo on MS+2.4 D, (B) Mature callus placed on regeneration medium, (C) Shoot emerging on regeneration medium, (D) single shoot placed on rooting medium, (E) Emerging rooting from a single shoot on rooting medium, (F) Wheat seedling transfer in the pot for acclimatization.

4. Conclusions

In the present study, the sterilization protocol has been more effective and less time-consuming for mature seeds and embryos. The positive effect of Bavistin on callus induction and shoot regeneration was also analyzed with the reduced fungal contamination observed in wheat tissue culture. Thus, optimized media displayed healthy callus induction and shot regeneration from mature embryos in the wheat variety HD2967 and could be utilized for other wheat germplasms.

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Conflicts of Interest

The authors declare no conflict of interest.

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