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Optimization of an efficient rapid regeneration of Indian wheat cultivars by callus induction and multiple shoot induction using mature embryos

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A B S T R A C T

Wheat is the second most produced staple food crop in world. An efficient method for in vitro micro propagation and genetic transformation of wheat are crucial for both basic and applied research. The present study was initiated with a view to develop a regeneration system in 4 genotypes of wheat cultivars HD2888, HD2329, DBW148, and DBW88 as a prerequisite to transformation. The mature embryos were excised from seeds and cultured on MS medium supplemented with high and low concentrations of cytokinins and auxins respectively. Callus induction was prominent with 4mg/L 2,4D with HD2888. The regeneration system was optimised and was prominent with 1mg/L kinetin by slowly reducing the concentration of 2,4D to 2mg/L. The MS medium containing 2 mg/L N6 -benzylaminopurine (BAP) and 0.5mg/L 2,4-D was found to be effective for multiple shoot formation in HD2932 cultivar that could produce 7 shoots per explants. The elongated shoots were separated and successfully rooted on half MS medium containing 0.2 mg/L indole-3-acetic acid (IAA) Callus mediated regeneration took approximately 36 days to regenerate where as multile shoot mediated regeneration took 20 days. Further studies will be taken up to check the transformation efficiency by transforming the desired gene through Agrobacterium mediated protocols.

Introduction

Abiotic and biotic stress are the major factors that are affecting the yield and nutritional quality of the crops especially wheat. Wheat is the second most produced staple food crop and india has the largest growing area of 31.47 million hectares and is the second largest producer of 86.53 million metric tons in the world. Global climate change is the important cause for yield loss in wheat. Hence, breaking the yield barrier and enhancing the abiotic stress tolerance are important goals of wheat improvement programs. Functional genomics of wheat is necessary to achieve these goals and requires availability of an efficient

transformation and regeneration protocol (Ali sarali *et al* 2013).

Wheat is considered as a recalcitrant crop. Due to this nature, several explants of wheat have been used for their regeneration and transformation capacity, including the immature embryos (Rakesh K et al 2017), mature embryos (Ali sarali et al 2013), shoot apical meristem (Ahmad et al. 2002), scutellar tissue (Becker et al. 1994), microspores (Ziemienowicz et al. 2012), protoplasts (Ahmed and Ságі 1993), inflorescence (Barro et al.1999) and leaf base (Haliloglu 2006). Immature embryos and inflorescence are the most frequently used explants due to their higher regeneration efficiency (Ziemienowicz 2014). Callus-mediated regeneration using mature and immature embryos explants have been reported (Rakesh K et al 2017). A good and efficient callus induction system in wheat highly depends on sterilization process, type of explants, genotypes, media composition and its pH, growth hormones, inducers and incubation conditions (Mamrutha et al. 2014).Mature embryos are available throughout the year whereas immature embryos are available only during 12–20 days post-anthesis limiting their application for in vitro culture and genetic transformation readily.

Most frequently used auxin to induce callus in wheat is 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin and potent herbicide, at a concentration of 1–2 mg/l. It is followed by dicamba (Ren et al. 2010) and picloram (Satyavathi et al. 2004). Till date, many regeneration media were optimized by adding cytokinins such as kinetin, 6-benzylaminopurine (BAP), thidiazuron (TDZ), zeatin along with auxins indole-3-acetic acid (IAA), 1-naphthaleneacetic acid (NAA), 2,4-D, picloram (Benderradji et al. 2012). Additionally, some inducers, such as CuSO4, AgNO3, were found to be useful to enhance the regeneration rate (Yu et al. 2008).

Genetic engineering opens opportunities to meet these challenges and allows introduction of novel desirable genes into host plants for the development of desired trait. However, efficient and rapid *in-vitro* regeneration is prerequisite for wheat improvement through genetic engineering and functional genomics approaches. Hence we reported a simple methodology for successful *in vitro* regeneration of wheat from mature embryos, developed based on combination of different growth regulators in four different wheat genotypes both by indirect callus mediated and direct multiple shoot induction mediated protocols.

Materials and Methods

Plant materials

Seeds of four wheat genotypes used for comparison of regeneration efficiency in this study, namely HD2888, HD2932, DWB148, DWB88 were obtained from AICRP, MARS, University of Agricultural sciences, Dharwad.

In vitro germination and explant excision

Mature embryos were used as explants for in vitro culture. Seeds were pre treated with 70% alcohol for 2 min followed by 3 to 5 rinses with sterile distilled water. The seeds were then surface sterilized with 4% sodium hypochlorite (v/v) with intermittent shaking for 15 minutes followed by 8 rinses with sterile distilled water. Seeds were then soaked overnight in sterile distilled water and embryos were excised from these imbibed seeds. The radical portion of mature embryo was slightly damaged and cultured with scutellum in contact with the medium to start initiation of callus.

Callus induction

Excised embryos were inoculated on petri

plates containing MS medium supplemented with 30 g/l sucrose, 3 mg/l casein hydrolysate and 3 mg/l proline (Ali sarali et al 2013) on four different concentrations ranging from 1-6 mg /L2,4D for optimization of regenerative callus. Calli were maintained in dark at 25ºC, and subcultured on fresh medium at one week interval. Later the callus was sub cultured on reduced concentration of callus 2mg/L 2,4D and then shifted to regenerative media.

In vitro regeneration

Induced calli were sub-cultured on MS medium supplemented with 30 g/l sucrose, 3 mg/l casein hydrolysate and 3 mg/l proline with the following two different hormonal concentrations for shoot organogenesis 1 to 5 mg/l kinetin. For in vitro regeneration calli were maintained on MS medium for 2 weeks at 25ºC temperature and 16/8 h light and dark cycles on petri plates. Later the Shoots were sub-cultured on the same medium at one week interval to test tubes containing the media.

Multiple shoot induction

For induction of multiple shoots, MS basal medium containing 30 g/l sucrose, 3 mg/l casein hydrolysate and 3 mg/l proline supplemented with different concentration of high level of BAP and low concentration of 2, 4-D was used. The cultures were kept in light at 22 ± 1 C in 16/8 h of light/dark cycle regime with sub culturing one in two weeks. Counting of multiple shoots was done for each explant after it was sub cultured twice. The main shoots were removed during sub culturing. Shoots which emerged from mature embryos directly without callus interphase were separated and transferred on rooting media.

Rooting and Hardening

The regenerated plantlets were transferred on MS media containing 0.2 mg/L IAA for rooting. Regenerated shoots were sub-cultured on half strength MS basal medium solidified with 8 g/ L agar. For root induction, the shoots were maintained for 7 days at 25ºC temperature, 16/8 h photoperiod of about 40-50 µE m–2s–1.Plantlets after rooting were hardened in liquid MS basal medium for 4 days. After primary hardening, plantlets were grown in mixture of the soil, peat and vermiculite (2:1:1), at 22°C under a 16/8 h. In the callus induced and direct shoot induction study the number of shoots per explants and percent of regeneration were recorded.

Results and Discussion

Callus induction and regeneration

The callus induction was tested on different concentrations 2,4,6 mg/L of 2,4,D and all tested callus induction media showed variable embryogenic callus induction efficiency in four Indian wheat varieties HD2888, HD2932, DWB148 and DWB88 along with different percentage of precocious germination.

The percent of callus induction with HD28888 showed 99% callus induction where as HD2932, DBW148 showed 89 and 86 % for mature embryos respectively with 4mg/L 2, 4, D. After one subculture of 7days the callus was sub cultured on 2mg/L 2,4,D in order to maintain the callus phase and also to induce the regeneration efficiency. The optimum induced callus was showed 99 % regeneration with MS media containing 1 mg/L kinetin and 2mg/L 2, 4, D. (Table.1; Fig1&2)

Multiple shoot induction and regeneration

The effect of different concentrations of 1-3 mg/L BAP alone and 0.5 mg/L 2, 4, D was tested. Optimization of multiple shoot induction was carried out to reduce the regeneration time of the wheat as callus induction mediated regeneration will take at least 1 to 2 months. Seven multiple shoots

was observed after 20 days of incubation at 22^{o}C, 16/8h light and dark incubation on 1mg/L BAP and 0.5 mg/L 2,4,D and HD2932 showed 100 % of regeneration compared to HD2888 (96%) and DBW88 (68 %) varieties. (Table.2:Fig.3). For HD2932 1mg/L BAP and 0.5 mg/L 2, 4, D is sufficient for initiating shoot regeneration. The shoots were sub cultured into shooting media containing 2mg/L kinetin and 0.5mg/L IAA. After a week of rooting the regenerated wheat was placed onto hardening material and the survived plants were transferred to pots in green house.

Both mature and immature embryos have been used extensively in tissue culture protocols, but mature embryos were found to be a better choice in comparison to immature embryos (Özgen *et al.* 1998). Immature embryos are better explant source when regeneration is considered, but they require time and growth facilities (Zale *et al.* 2004) whereas mature embryos are available throughout the year. Mature embryos can either be dissected (Yu *et al.* 2008) or used directly (Özgen *et al.* 1998).

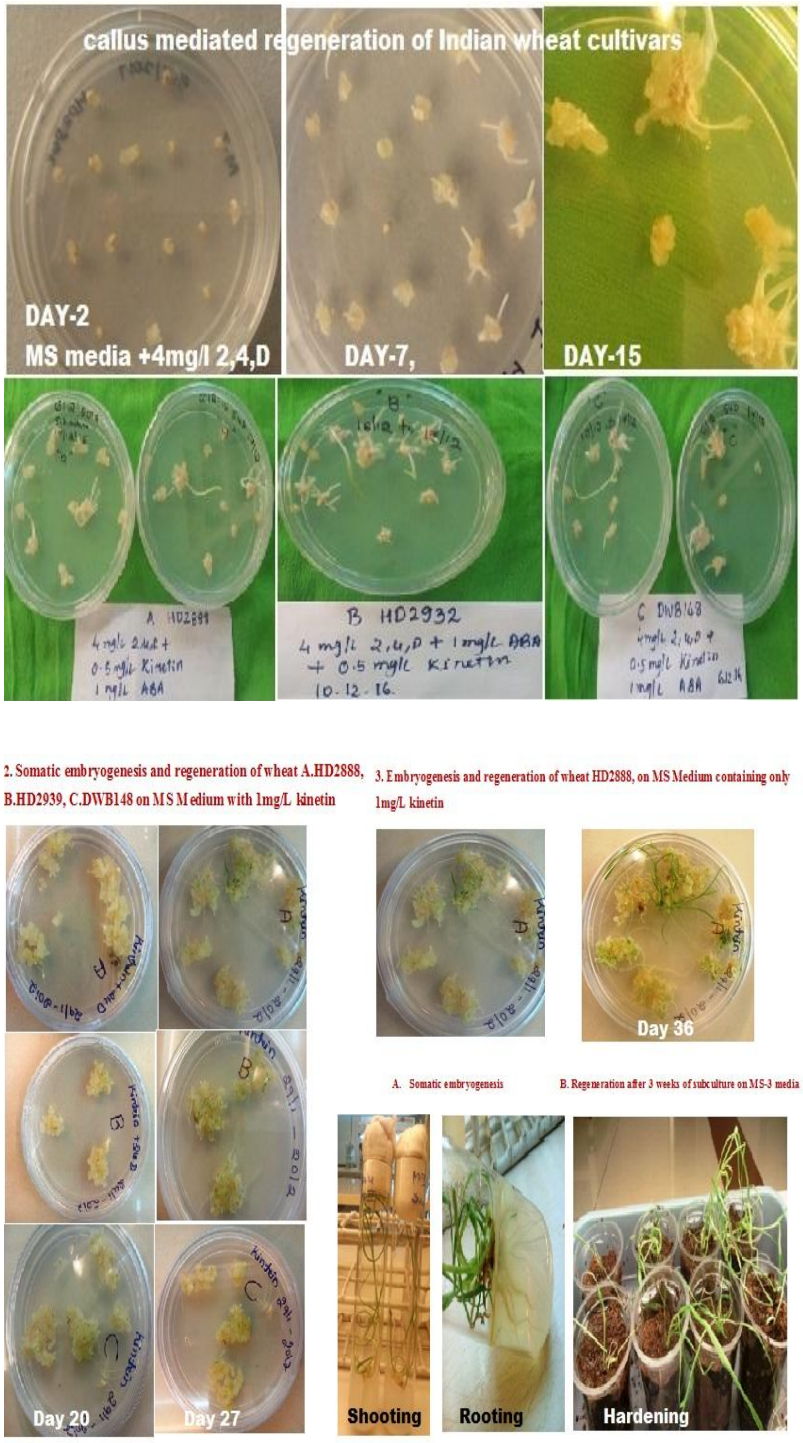
Table.1 Different Combination of Growth Regulator in MS Media used for Callus induction and Regeneration.

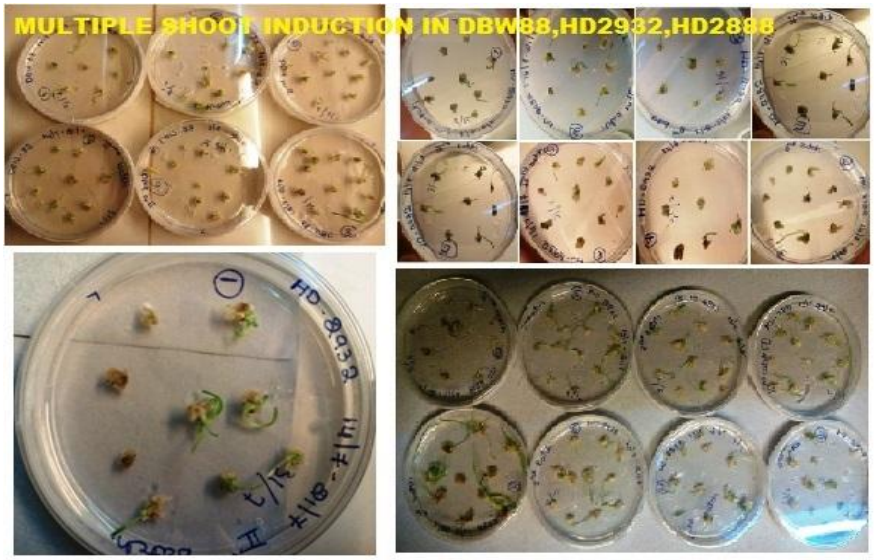
Wheat Variety	Concentrations of 2,4,D (mg/l)	Percent Induction (%)	Combinations of 2,4,D and Kinetin (mg/l)		Percent Regeneration (%)
HD2888	2	95	2	0.5	80
HD2932	2	86	2	0.5	76
DBW148	2	60	2	0.5	55
HD2888	4	99	2	1	100
HD2932	4	96	2	1	89
DBW148	4	86	2	1	65
HD2888	6	75	2	2	85
HD2932	6	63	2	2	70
DBW148	6	45	2	2	55

Table.2 Different Combinations of Growth Regulators in MS Media used for Multiple Shoot Induction

Wheat Variety	Combinations of BAP and 2,4,D (mg/l)		Shoot induced (nos)	Percent regeneration (%)
HD2888	1	0.5	5	89
HD2932	1	0.5	7	100
DBW88	1	0.5	3	68
HD2888	2	0.5	4	89
HD2932	2	0.5	5	95
DBW88	2	0.5	3	48
HD2888	3	0.5	2	62
HD2932	3	0.5	2	65
DBW88	3	0.5	2	54

Fig.1


callus mediated regeneration of Indian wheat cultivars
DAY-2
MS media +4mg/l 2,4,D
DAY-7,
DAY-15
A. HD2888
4 mg/l 2,4,D + 0.5 mg/l Kinetin + 1 mg/l ABA
B HD2932
4 mg/l 2,4,D + 1 mg/l ABA + 0.5 mg/l Kinetin
10-12-16
C DWB148
1 mg/l ABA
2. Somatic embryogenesis and regeneration of wheat A.HD2888, B.HD2939, C.DWB148 on MS Medium with 1mg/L kinetin
3. Embryogenesis and regeneration of wheat HD2888, on MS Medium containing only 1mg/L kinetin
Day 36
A. Somatic embryogenesis
B. Regeneration after 3 weeks of subculture on MS-3 media
Day 20
Day 27
Shooting
Rooting
Hardening



Second step is the optimization of the medium on which maximum plantlets are regenerated. Indole-3-Acetic Acid (IAA), 6-BenzylAminoPurine (BAP) and Kinetin were the most extensively used hormones (Haliloglu, 2006). But others can also be used like Thidiazuron (Xueyan *et al*. 2000). There are several drawbacks associated with these growth regulators such as longer incubation period resulting in the loss of explants viability for regeneration and high cost etc.

The callus is a rapidly proliferating undifferentiated mass of cells, which can be obtained by culturing explants on nutrient media containing specific growth hormones (Naqvi *et al.,* 2002). Media composition-mainly the hormonal balance-is an important factor influencing *in vitro* culture initiation and plant regeneration from embryos (Jiang *et al.,*1998) The auxin 2, 4-dichlorophenoxy acetic acid (2, 4-D) alone or in combination with cytokinins, is widely used to enhance callus induction and maintenance (Castillo *et al.,* 1998). In tissue culture of cereals including wheat, 2,4-D is often used for callus induction, and different combinations of auxin such as napthaleneactic acid (NAA) and indoleacetic acid (IAA) and cytokinin were used for stimulating plant regeneration from calli (Bhaskaran *et al.,*1990).

The present study is on the plant growth promoters which influence on callus induction and regeneration to find out the optimum concentrations using mature embryos in Indian wheat cultivars. Studies have reported the effect of growth regulators on callus induction and regeneration in wheat (Chauhan et al. 2007). But these studies were carried out in limited number of wheat genotypes and with less number of PGR combinations in the media. Earlier reports, claims 2,4-D has optimum callus induction capacity in wheat (Yu et al. 2008). However, our results indicated that 4 mg/L 2,4-D has the fastest rate of callus induction. Shoot regeneration is another crucial step after callus induction in tissue culture. BAP and kinetin were tested at different concentration and combinations. The absence of 2,4-D in regeneration medium showed the shoot induction as well as the root induction. The root induction suppressed to a large extent by supplying 2,4-D alone in regeneration medium or with other growth regulators, only shoot induction occurred. It was evident that the inclusion of 2,4-D in combination with cytokinins is valuable for regeneration. The present study results

correlate with Chauhan et al. 2007. Overall, the standardized regeneration showed 100% regeneration efficiency irrespective of the genotype both by callus mediated and multiple shoot induction by mature embryo as explants.

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