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Optimization of regeneration protocol and prospecting spectinomycin resistance in barley (*Hordeum vulgare* L.) cv Haider-93

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Immature zygotic embryos from spring barley cv. Haider-93 were used to induce somatic embryogenesis. The type of the explant, the level of 2,4-dichlorophenoxyacetic acid (2,4-D) and handling of calli during subculture are critical factors to obtain maximum number of regenerants. Different concentrations of 2,4-D (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/L) and Kinetin (0.5, 1.0, 1.5, 2.0 mg/L) were used for callus induction and shoot initiation, respectively. Use of immature embryos having damaged axis as explants revealed a pronounced gradient of callus formation on Murashige and Skoog (MS) medium supplemented with 2.5 mg/L of 2,4-D and maximum regeneration response at 1 mg/L kinetin. Regenerated shoots were rooted on MS medium supplemented with 1 mg/L IAA. A kill curve was developed to find out the sensitivity level of barley cells to spectinomycin, a broad-spectrum antibiotic. This study fulfils an antique prerequisite of a reproducible regeneration system required for the improvement of barley via genetic engineering and also falls under, "Establish Good Health and Well-Being" Sustainable Development Goals of United Nations Organization.

Key words: 2,4-Dichlorophenoxyacetic acid, indole acetic acid, kinetin, antibiotic resistance, spectinomycin, Barley 53.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is the fourth most important cereal crop in the world after wheat, maize and rice (Rostami et al., 2013). Barley is a self-pollinated crop belonging to family Poaceae and genus *Hordeum* (Hu et al., 1983) which evolved from *Hordeum spontaneum* (Nevo, 1992). Genus *Hordeum* comprises 25 to 30 species distributed all around the world.

Barley is a diploid species with two sets of chromosomes (2n=2x=14) and base number is seven. Barley is an annual, short duration crop and additionally

have good adoptability, that is, it can be cultivated from 42° latitude South to 70° latitude North. Seed is an edible part of barley plant, formed in the palea of spikelet. Seeds of barley are harder than any other cereal. Barley can be cultivated in all extreme climates including arctic, subarctic and dry areas (Guo, 1987; Baik and Ullrich, 2008), but high humidity affects its growth (Smith, 2004). Barley grain is mostly used in malt production and animal feed because of its high nutritional value which is 95% more than that of maize. Malt from barley is used in beer making, analytical grade alcohol production, whisky, and syrups. About 1000 million people in Tibetan cuisine and Medieval Europe use barley as staple food. Mostly 85% cultivated barley is used as animal feed while the remaining is utilized in malting and brewing industry for human consumption.

Barley has high nutritional value possessing maximum value of vitamins, minerals, fiber and bioactive compounds namely β-glucans, tocopherols, tocotrienols and phenolic compounds that are an excellent source of antioxidants for disease prevention (Baik and Ullrich, 2008; Gallegos-Infante et al., 2010). High content of soluble fibers found in Barley assist in the prevention of constipation and colon cancer whereas insoluble fibers aid in lowering blood cholesterol leading to the prevention of cardiovascular diseases (Ötles and Ozgoz, 2014). Barley has high content of β-glucans than any other cereal (Zhang et al., 2003), containing numerous vitamins like thiamine (vitamin B1), niacin (vitamin B3), and minerals such as iron, copper, magnesium, phosphorus, zinc, and selenium (Pins and Kaur, 2006). Barley can store 15% proteins (by dry weight) for up to 10 years (Tanasienko et al., 2011). In some areas like California, barley is used for hay production.

There are some oriental countries, like Korea, Japan, China, and Himalayan using naked barley for cooking, grinding, and beverages production.

Cultivation area of barley is declining day by day due to urbanization and different stresses such as biotic and abiotic stress. The only possible way to improve yield of barley is by producing new varieties with high resistance against biotic (El-Sappah et al., 2021a) and abiotic stress factors by employing robust genetic engineering and gene silencing techniques such as CRISPR/Cas9 and RNAi (Abbas et al., 2020; El-Sappah et al., 2021b). An *in vitro* reproducible regeneration system including dedifferentiation, redifferentiation, regeneration, and organogenesis is the prerequisite for genetic transformation against biotic and abiotic stresses. Tissue culture is also helpful in seed cost reduction, better crop improvement and seed importation. Development of tissue culture and regeneration protocol is vital for application of biotechnological tools for genetic improvement, soma clonal variants recovery, transgenic production, clonal propagation, pathogen free plants production and preservation of germplasm (Ehsanpour and Jones, 2000; Ganeshan et al., 2003). Antibiotic resistance genes are antique part of vectors for identification of successful transgenic events. Antibiotic along with Green Fluorescent Protein (GFP) have proved most suitable selection system (Khan and Maliga, 1999). Noticeably, some plants naturally harbor resistance against antibiotics. Therefore, it is mandatory to undermine lethal dose of a broad-spectrum antibiotic in barley.

In this study, immature embryos of barley were cultured on medium supplemented with different concentrations of hormones for callus induction, proliferation and regeneration. Barley seeds were also cultured on MS medium supplemented with different concentrations of broad spectrum antibiotic spectinomycin to prospect resistance inheritance of barley. Spectinomycin is used as conditional positive selectable marker for selection of transgenics (Mustafa and Khan, 2012) which causes bleaching by inhibiting chlorophyll biosynthesis (Oreifig et al., 2004). The present study was conducted to provide precise supportive bench for genetic transformation for the improvement of cereal crop barley against biotic and abiotic stress and increasing production.

MATERIALS AND METHODS

Explant for callus induction

Seeds of barley (*H. vulgare* L.), commercial cultivar Haider-93 were collected from Ayub Agricultural Research Institute (ARRI), Faisalabad. Seeds were cultivated by sowing in pots filled with mixture of sand, silt and organic matter at a ratio of 2:2:1 and placed in green house. At flowering, spikes were covered with brown bags to avoid cross pollination and immature seeds (DAP=15) were used for callus induction and regeneration. Immature seeds were surface sterilized with 70% (v/v) ethanol for 1 min, and then with 3% sodium hypochlorite (NaOCl) for20 min and finally five washings with sterile double distilled deionized H_2O were given (Salama et al., 2013; Abbas et al., 2016). Immature embryos were dissected and cultured in Petri plates containing MS medium supplemented with different concentrations of 2,4-D. Cultures were maintained at 26±2°C in light under a 16 h photoperiod.

Medium for callus induction

The basal medium for callogenesis, regeneration and root formation was MS medium (Murashige and Skoog, 1962). Stock

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Table 1. Different concentrations of 2,4-D in culture medium used for callus induction.

S/N	Treatments	Media compositions
1	T_0	MS0
2	T_1	MS + 0.5 mg/l 2,4-D
3	T_2	MS + 1.0 mg/l 2,4-D
4	T_3	MS + 1.5 mg/l 2,4-D
5	T_4	MS + 2.0 mg/l 2,4-D
6	T_5	MS + 2.5 mg/l 2,4-D
7	T_6	MS + 3.0 mg/l 2,4-D

Table 2. Plant regeneration medium for immature embryos derived calli of cv. Haider-93.

S/N	Treatment	Media composition
1	T_0	MS0
2	T_1	MS + 0.5 mg/L kinetin
3	T_2	MS + 1.0 mg/L kinetin
4	T_3	MS + 1.5 mg/L kinetin
5	T_4	MS + 2.0 mg/L kinetin

solutions of MS salts, vitamins and growth hormones were formed in double distilled deionized H_2O. As a source of C, sucrose was added in medium at a concentration of 30 g/L. pH of culture medium was adjusted to 5.8 and solidified with 8 g/L agar. Medium was autoclaved at 121°C at 15 psi for 20 min, aliquots (30 ml) of each medium were poured in the Petri plates and wrapped with clink film and incubated at room temperature to examine contamination (Cheng et al., 2021). MS plane medium supplemented with different concentrations of spectinomycin was used to examine resistance inheritance of barley.

Immature embryos were excised under aseptic conditions in laminar flow hood with the help of scalpel; embryogenic axis were damaged with the help of sterilized surgical blade and cultured on MS medium (Murashige and Skoog, 1962) supplemented with various concentrations of 2,4-D to optimize callus induction (Table 1). Plates containing cultured embryos were incubated at 23±2°C under dark condition. Calli were sub-cultured on the same medium after a two-week interval for proper nutrient supply. For the determination of callus growth rate, calli were transferred to a sterile Petri dish and the weight of calli present in a plate was measured in aseptic conditions. Afterwards, callus pieces were re-transferred to callus culture medium. This measurement was repeated every week for 8 weeks.

Medium for regeneration

Half strength MS medium supplemented with different concentrations of kinetin (0.5, 1.0, 1.5, 2.0 mg/L) (Table 2) was used for regeneration of plantlets by incubating callus in growth room at 25±2°C, 16 h light and 8 h dark (Abbas et al., 2020). After dark incubation period, calli were transferred onto Petri dishes containing regeneration medium (MS) supplemented with kinetin for shooting. After 4-, 8- and 12-weeks data was collected as the number of shoots regenerated per explants. For shoot regenerating explants, numbers of shoots per each explant were also recorded (Ahloowalia, 1982).

Rooting of *in vitro* regenerated shoots

Shoots of specific height (1.5 cm) were removed from medium containing kinetin for regeneration and transferred in 400 ml Magenta boxes containing 50 ml of half-strength MS medium supplemented with 1.0 mg/L IAA for rooting. Magenta boxes were incubated at 25±2°C in growth room. Regenerated plants were removed from the magenta boxes after they reached the expected developmental stage. The roots were then completely cleaned with distilled water after being properly rinsed with tap water to remove the media.

Plants were transferred in pots containing sterile clayey soil and sand (50:50), covered with polythene bags and placed in growth chamber at 21±1°C and 13% relative humidity. After 15 days of acclimation, plants were shifted in greenhouse and fertilized with NPK fertilizers mixture with a ratio of 17:17:17 (Aguado-Santacruz et al., 2011).

Prospecting antibiotic resistance

An effective antibiotic with its appropriate concentration is requisite for identification of successful transformation system (Mustafa and Khan, 2012). After sterilization, barley seeds were transferred onto MS0 medium (Murashige and Skoog, 1962) with various concentration of antibiotic (Table 7) to examine the resistance inheritance of barley (*H. vulgare* L). Magenta boxes were visited regularly to monitor the effect of antibiotic on the germinated plantlets of barley.

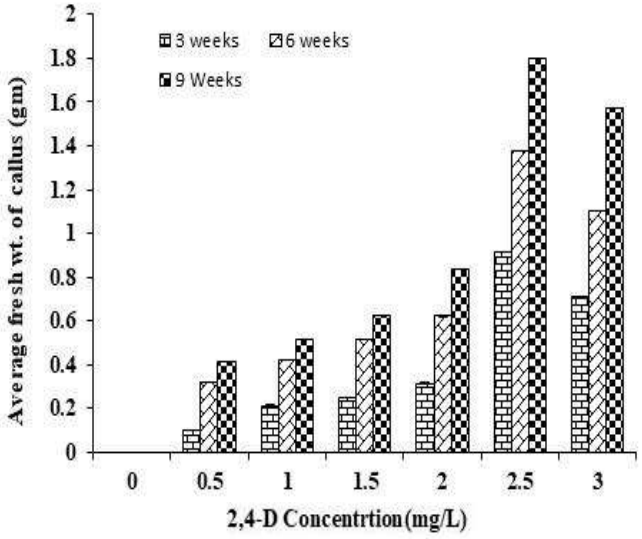

Figure 1. Different concentrations of 2,4-D for callus induction in immature embryos of barley.

RESULTS

Callus induction on MS + auxin (2,4-Dichlorophenoxy acetic acid)

Efficient and reproducible *in vitro* regeneration techniques leading to the production of plants from cultured tissues are of prime importance to clonal propagation and other genetic engineering approaches of barley plants. Callogenesis from immature embryos (DAP=15) of barley (*H. vulgare* L.) cultured on MS medium supplemented with different concentrations of 2,4-D was initiated within 3 to 4 days (Figure S1) of culture and maximum callus induction frequency (95%) was achieved on MS medium supplemented with 2,4-D at the concentration of 2.5 mg/L (Figure 1). Further increase in 2,4-D negatively affected callus induction and its weight (Table 3). For three weeks after incubation, callus was observed for proliferation purpose to get appropriate mass. We observed shoots initiation in some calluses which were removed with the help of scalpel in aseptic conditions (Figure S2). After three weeks of incubation, efficient callus induction frequency and size was calculated by applying the following formula.

Callus induction (%) = No. of embryos product calli / Total No. of embryos cultured × 100

The callus mass and frequency were recorded after every three weeks of inoculation for a period of 9 weeks, at maximum. Callus induction, proliferation frequency and its weight varied with respect to the concentration of 2,4-D used (Table 3).

Morphological characterization of callus

Calli induced in immature embryos were characterized for their amount, color and texture induced at various concentrations of 2,4-D (Table 4). After 8 to 9 weeks of incubation, friable calli were developed which were characterized morphologically (Figure 2). No callus induction (-) was observed on MS plane medium; poor (+) amount, creamy, whitish, creamy white, compact and watery callus were induced on MS medium supplemented with 0.5, 1.0 and 1.5 mg/L of 2,4-D, respectively. Good (+ + +) amount, creamy white and friable callus were induced on MS medium supplemented with 2.0 mg/L of 2,4-D, while best (+ + + +) and highest amount of callus with creamy white color and friable texture were induced on MS medium supplemented with 2.5 mg/L of 2,4-D. Finally, satisfactory (+ +) amount with creamy white color but watery callus were induced on MS medium supplemented with 3.0 mg/L of 2,4-D (Table 4).

Callus to plant regeneration on MS + kinetin medium

Calli were observed for length and number of shoots initiation after 4, 8 and 12 days. The lengths and numbers of newly regenerated shoots from different calluses cultured for shoot initiation on MS medium supplemented with the aforementioned concentrations of kinetin were varied (Figure S3). Within 4 days, shooting primordial became visible and after 12 days, maximum number of shoots was regenerated from calluses (7-11 shoots/ callus) with lush green color (Figure 3 and Table 5). Maximum numbers of shoots (11) with lush green color and appropriate length (1.4 cm) were observed on MS medium containing 1.0 mg/L of kinetin (Table 5). Highest shoot regeneration efficiency was measured on MS medium supplemented with 1.0 mg/L of kinetin calculated by applying the following formula (Table 5).

Embryogenic efficiency (%) = No. of shoots producing calli / No. of embryos incubated × 100

Rooting and acclimation of regenerated shoots

Cluster of roots were initiated within 4 days after transferring regenerated shoots on rooting medium and significant number of roots were developed within 12 days (Figure 4). Maximum rooting frequency was observed on MS medium supplemented with 1.0 mg/L IAA calculated by the following formula (Table 6).

Regeneration efficiency (%) = No. of root producing calli / No. of incubated embryos × 100

Plants with developed roots were shifted to half liter pots

Table 3. Different concentrations of 2,4-D for callus induction in immature embryos of barley (*Hordeum vulgare* L).

Concentration of 2,4-D (mg/L)	Average fresh weight (g)			Total cultured embryos	Callus induction frequency (%)
	3 weeks	6 weeks	9 weeks		
0.0	Control	Control	Control	90	0
0.5	0.101 ± 0.001	0.321 ± 0.0005	0.414 ± 0.0005	90	2
1.0	0.210 ± 0.005	0.422 ± 0.0005	0.513 ± 0.0005	90	9
1.5	0.251 ± 0.0005	0.513 ± 0.0005	0.622 ± 0.0005	90	22
2.0	0.315 ± 0.0005	0.621 ± 0.0005	0.837 ± 0.0005	90	58
2.5	0.914 ± 0.0005	1.374 ± 0.0005	1.798 ± 0.0005	90	95
3.0	0.712 ± 0.0005	1.103 ± 0.0005	1.571 ± 0.0005	90	44

Table 4. Morphological characterization of callus of barley cv. Haider-93.

Treatment (MS+2,4-D)	Callus amount	Callus morphology/Explant callus color	Callus texture
0	-	-	-
0.5	+	Creamy	Compact
1.0	+	Whitish	Compact
1.5	+	Creamy White	Watery
2.0	+ + +	Creamy White	Friable
2.5	+ + + +	Creamy White	Friable
3.0	+ +	Creamy White	Watery

(-) No callus; (+) Poor callus; (+ +) Satisfactory Callus; (+ + +) Good Callus; (+ + + +) Best Callus.

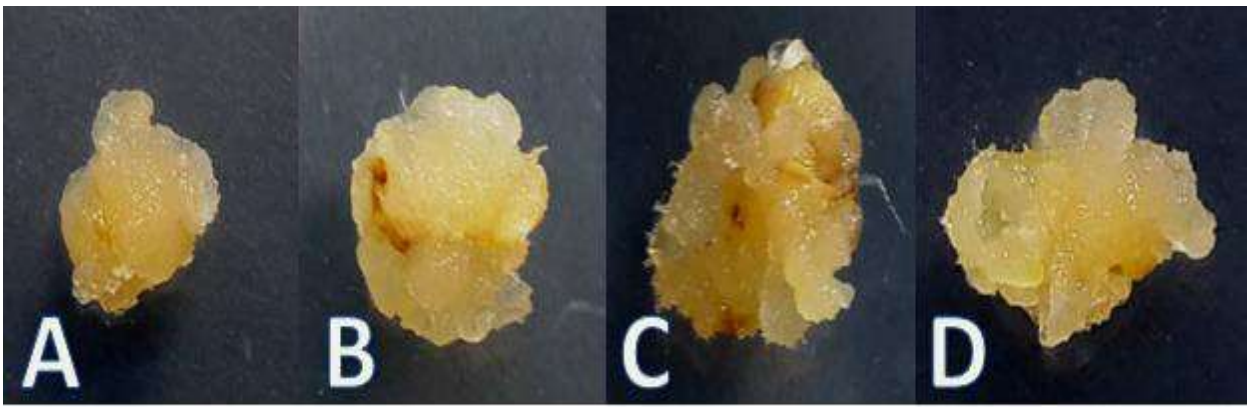

Figure 2. Callus formed on MS medium supplemented with 2.5 mg/L 2,4-D (A) after 3 weeks, (B) 6 weeks, (C) 7 weeks, and (D) 9 weeks.

filled with soil and sand (50:50) along with organic matter, covered with transparent polyethylene bags and placed in a growth chamber at 21±1°C, at a 13% relative humidity and at a photon flux of 70 µmol m^{-2} s^{-1} for hardening.

After 2 weeks, pots were shifted to room temperature and regularly fertilized with NPK (17:17:17) (Peng et al., 2019).

Prospecting spectinomycin resistance in barley

Change in color or chlorosis of leaves was observed indicating spectinomycin had not affected pigmentation in chlorophyll and barley exhibited strong resistance. Although root and shoot length varied on medium supplemented with different levels of spectinomycin, all

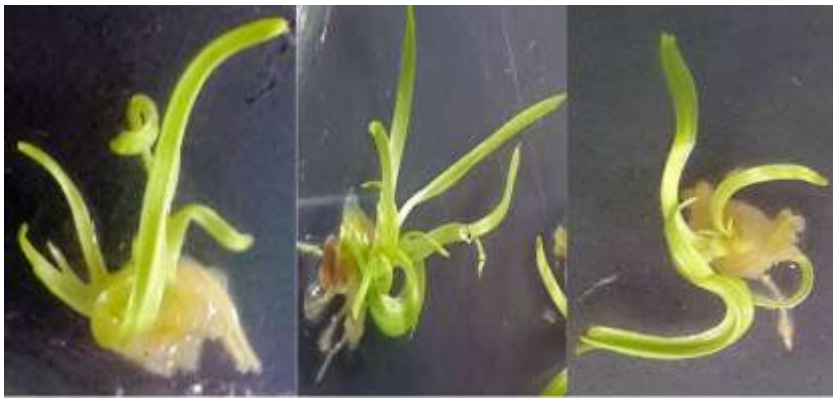

Figure 3. Regenerated plantlets from callus induced in immature embryos of barley (*Hordeum vulgare* L).

Table 5. Effect of kinetin on regeneration of calli of cv. Haider-93

S/N	Conc. of kinetin (mg/L)	No. of shoots			Shooting efficiency (%)
		4 days	8 days	12 days	
1	0.5	2 ± 0.57	5 ± 1.73	7 ± 1.15	67
2	1.0	4 ± 1.15	8 ± 0.73	11 ± 0.57	95
3	1.5	3 ± 1.15	4 ± 1.15	8 ± 1.15	82
4	2.0	2 ± 0.57	4 ± 0.57	6 ± 1.73	34

Figure 4. Effect of IAA on rooting of regenerated plants.

were found resistant to spectinomycin (Figure 5), corroborating that barley is naturally resistant to spectinomycin (Figure S4).

DISCUSSION

Tissue culture and regeneration of barley (*H. vulgare* L.)

Table 6. Effect of IAA on rooting of regenerated plantlets.

S/N	Conc. of IAA (mg/L)	Average No. of roots			Rooting efficiency
		4 days	8 days	12 days	
1	0.5	2 ± 0.57	3 ± 0.57	7 ±0.57	53
2	1.0	3 ± 0.57	6 ± 1.73	9 ±0.57	95
3	1.5	2 ± 0.57	5 ± 1.73	8 ± 2.3	79
4	2.0	1 ± 0.57	3 ± 1.15	6 ± 0.57	42

Table 7. Effect of spectinomycin on genetic of cv. Haider-93.

Conc. of Spectinomycin (mg/L)	Shoot length (cm)	Root length (cm)	No. of tillers	Pigmentation
0	20.5 ± 0.28	5.5 ± 0.28	1 ± 0.57	+ + + +
25	21.3 ± 0.34	6.5 ± 1.28	1 ± 0.0	+ + + +
50	22.5 ± 0.57	7.5 ± 0.28	2 ± 0.57	+ + + +
100	21.3 ± 0.17	5.5 ± 1.28	1 ± 0.57	+ + + +
200	22.1 ± 0.31	7.5 ± 0.28	2 ± 0.57	+ + + +
300	22.1 ± 0.31	6.1 ± 0.11	2 ± 0.57	+ + + +
400	12.3 ± 0.17	2.1 ± 1.11	1 ± 0.57	+ +
500	18.5 ± 0.28	7.6 ± 0.11	2 ± 0.0	+ + +
1000	20.1 ± 0.31	9.1 ± 0.05	1 ± 0.57	+ + +

Chlorophyll pigmentation (+ +) = 25-50%, (+ + +) = 50-75%, (+ + + +) = 75-100%.

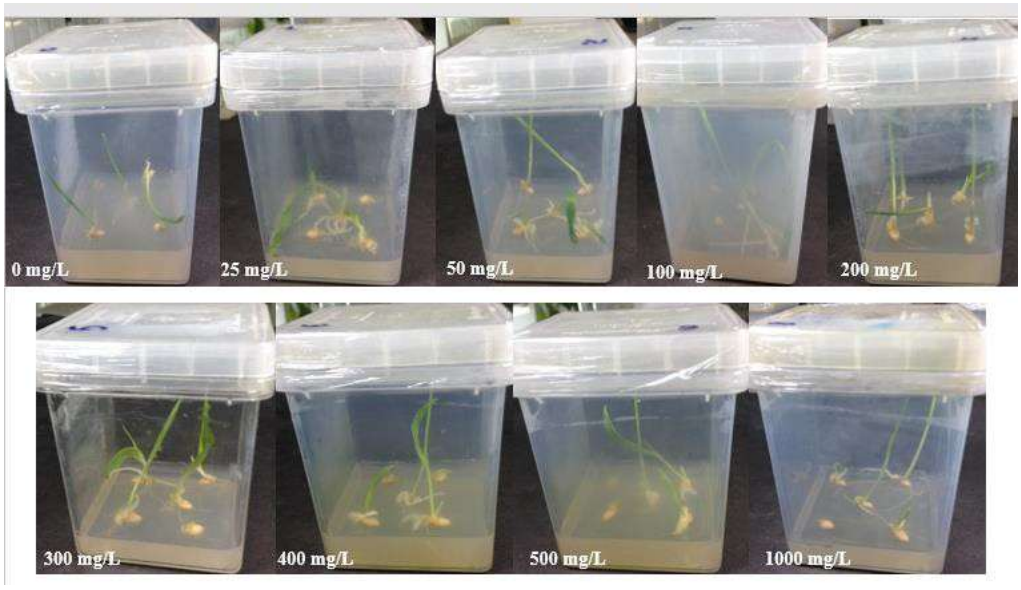

Figure 5. Effect of spectinomycin on number of tillers, shoot and root length of barley and bar = 5 cm.

are prerequisite for genetic transformation against biotic and abiotic stress resistance and biofortification. *In vitro* callus culture and regeneration of cells and tissues have broken the seasonal boundaries for production and manipulation of different crop species. Tissue culture has gained considerable attention in the recent years due to

its wide application in plant biotechnology for genetic improvement of plants. Various explants have extensively been used in establishing tissue culture protocols, such as anthers, ovaries, mature and immature embryos, etc. However, immature embryos have been reported to respond efficiently to callus induction and regeneration in comparison with mature embryos (Özgen et al., 1998; Zale et al., 2004). Using mature embryos, on the other hand, exhibits round-the-year availability which is a major advantage but low regeneration frequency is a serious drawback (Özgen et al., 1998; Yu and Wei, 2008).

Genetic improvement by both direct and indirect method is dependent on reproducible *in vitro* callus induction and regeneration system. Weights of calli induced by using immature embryos (DAP=15) as an explant on MS medium supplemented with 0.5, 1.0, 1.5, and 2.0 mg/L of 2,4-D measured as 0.414, 0.513, 0.622 and 0.837 g, respectively (Ahloowalia, 1982). Maximum callus weight was measured to be 1.798 g on MS medium containing 2.5 mg/L of 2,4-D as compared to maximum callus induction in wheat immature embryos at 2 mg/L of 2,4-D (Ozias-Akins and Vasil, 1982). However, further increase in 2,4-D concentration showed a decrease in callus weight, that is, 1.571 g on medium containing 3 mg/L of 2,4-D and so on. The present study revealed specific trend in callus induction on increasing concentration of 2,4-D. Callus weight increased with increasing 2,4-D concentration up to 2.5 mg/L. Further increase in 2,4-D resulted in decrease in average fresh weight of calli. Graphical representations of the relationship between various concentrations of 2,4-D and average fresh weight are illustrated in Table 5. More friable, embryogenic, creamy callus was observed at lower 2,4-D concentrations up to 2.5 mg/L in wheat (Ozias-Akins and Vasil, 1982). At the highest concentration, callus was more compact and yellowish in color with watery appearance (Goldstein and Kronstad, 1986).

All antibiotics have unique mode of action and exhibit different stringency against various plant species (Wilmink and Dons, 1993). Streptomycin is the most stringent antibiotic in its mode of action being tested while spectinomycin had no effect on barley (*H. vulgare* L.) regeneration because barley is a monocot. Literature suggests that streptomycin and spectinomycin irreversibly binds to the 30S ribosome and freezes the 30S initiation complex (30S-mRNA-tRNA), so that no further translation initiation occurs (Qian et al., 2012). Application of antibiotic predominantly resulted in slowdown of protein synthesis and hence induced misreading of the mRNA. Chlorosis occurs when calli are exposed to streptomycin due to severe halt in protein biosynthesis (Svab et al., 1990). Noticeably, aadA gene is responsible for conferring resistance against spectinomycin and streptomycin which poses no threat to humans or environment, thus can be potential selectable marker gene for plant transformation and also approved by European Food Safety Authority (https://www.efsa.europa.eu/en).

Immature embryos showing callus formation frequency up to 95% resulted in higher regeneration efficiency on medium supplemented with 1 mg/L kinetin is in line with previous study (Goldstein and Kronstad, 1986). The regenerated shoots were rooted on MS medium supplemented with 1 mg/L IAA (Murashige and Skoog, 1962) and acclimation was also performed. Dicotyledonous crop species upon exposure to spectinomycin show sensitivity and bleaching effect which are being successfully transformed for both nuclear and plastid transgene transformation (Day and Goldschmidt-Clermont, 2011). Spectinomycin resistance gene aadA is a valuable selection marker that is being used for the transformation of many plant species, that is, tomato, tobacco, brinjal and potato (Singh et al., 2010). In conclusion, spectinomycin is not a useful selectable marker in case of barley transformation system. The present study also revealed that barley has strong resistance against antibiotic spectinomycin up to 1000 mg/L used in culture medium.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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SUPPLEMENTARY MATERIAL

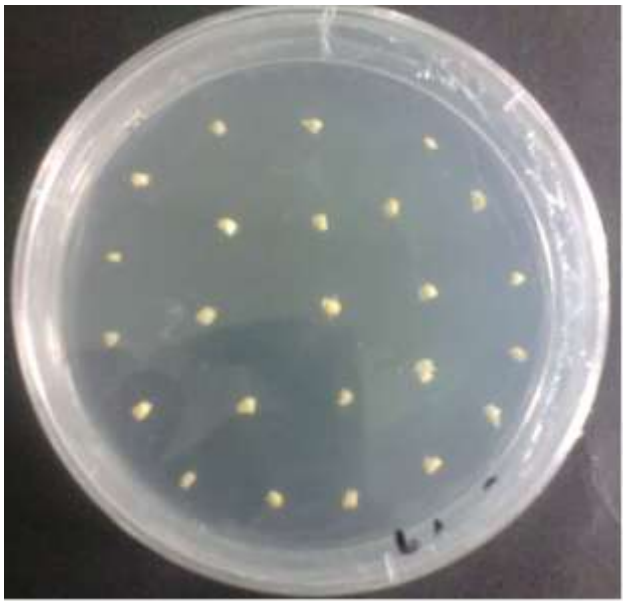

Figure S1. Callogenesis in immature embryos of barley cultured on MS + 2,4-D was started within 3-4 days.

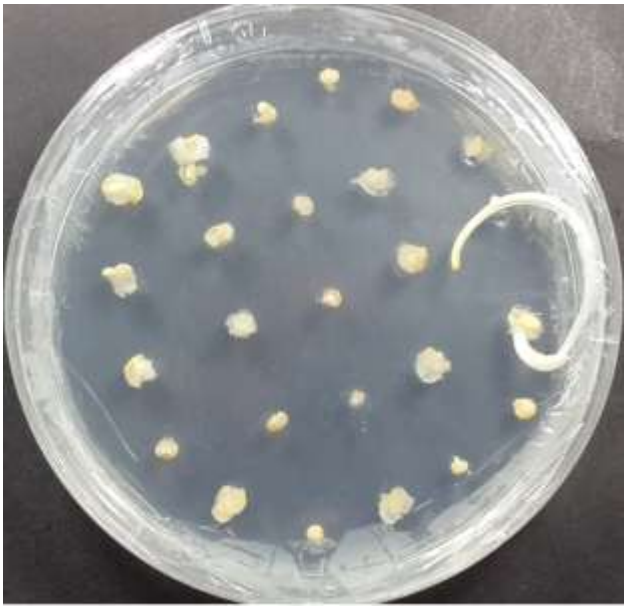

Figure S2. Shoots emerged on MS + 2,4-D removed with the help of scalpel.

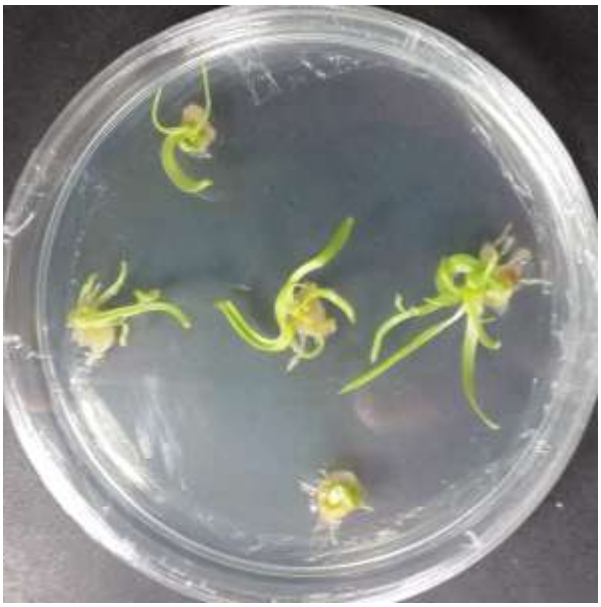

Figure S3. Rooting initiation on MS + Kinetin.

Figure S4. Prospecting antibiotic resistance in barley on MS + Spectinomycin.