Bulgarian Journal of Agricultural Science, 25 (No 6) 2019, 1099–1106

In vitro regeneration and *Agrobacterium* mediated transformation of Turkish commercial barley (*Hordeum vulgare* L.)

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Abstract

Hazrati, N., Unver, S., Hasanzadeh, M., Nofouzi, F., Khawar, K. M. & Ergul, A. (2019). *In vitro* regeneration and *Agrobacterium* mediated transformation of Turkish commercial barley (*Hordeum vulgare* L.). *Bulgarian Journal of Agricultural Science, 25* (6), 1099–1106

The aim of the work was to create *Agrobacterium*-based transformation method for barley and use this technology to produce stable transgenic plants so; the sterilized seeds of the barley *Özen* genotype were primed in 4 different nutrient media. Regeneration test was then carried out using embryo explants of germinated seeds in medium containing seven hormone levels of NAA (naphthalene acetic acid) + BAP (benzyl amino purine). According to the results, priming factor was significant ($P<0.01$) for all measured traits except for root number per explant and hormone factor was significant for all measured traits except for leaf area trait. According to the mean comparisons, priming medium containing 20 mg/l BAP was selected as the best one. The nutrient medium containing 0.1 mg/l NAA+0.45 mg/l BAP showed the highest plant growth factors. To investigate genetic transformation of barley *Özen* genotype, zygotic embryos (control embryos and embryos treated with cold treatment for 24 hours on MS medium) were treated with *Agrobacterium tumefaciens* GV 2260 strain harboring p35 GUS-INT plasmid followed by co-cultivation for 24 and 48 hours and then explants transferred to the selection medium. Survived plants were planted in peat soil and the leaves of developing and acclimated plants were subjected to GUS histochemical analysis and polymerase chain reaction (PCR). The highest yields were obtained in the explants subjected to 24 hours cold treatment followed by co-cultivation for 48 hours so that, the percent of green plants (17.18%), percent of plants with positive GUS (15.6%) and percent of plants with positive PCR (14.06%), had the highest values.

Keywords: transformation, *Agrobacterium tumefaciens,* GUS, PCR, Barley

Introduction

Barley, wheat, triticale and corn play important role in nutrition of human beings. Barley (*Hordeum vulgare* L.) is a self-fertilized productive member of the Gramineae family (Dunwell, 1986) and is used as a genetic model system because of its true diploid feature and its genome similarity to other cereals. One of the important aims of biotechnology is the production of characteristically high quality plant varieties by means of tissue culture and gene transformation techniques. In plants, efficient genetic transformation system for functional genomic studies is one of the key strate-

gies (Bakshi et al., 2011). Cereals *are considered* as important goals for genetic manipulation techniques application. Manipulation work is usually done with the *Agrobacteria* tool, and unfortunately most monocotyledons are not natural hosts for *Agrobacteria*. The natural infection capacity of bacterium in *Agrobacterium*-mediated transformation is used to transfer genes to plant cells (Chilton, 2001; Somers et al., 2003; Sangwan et al., 2010) and this approach is used for plant breeding and plant development. Since the mid-1990s, genetic engineering has provided faster and more direct modifications to cereals or provided new areas for the introduction of agronomically beneficial properties (Kumlehn et al., 2009; Hensel et al., 2009). The identification of a large number of transgenic plants occurred in 1994. The first report of fertile transgenic plants was presented by Wan & Lemaux (1994). In this report, immature embryos were transformed by microprojectile bombardment method. Subsequently, *Agrobacterium*-based transformation occurred in immature embryo explants in barley (Tingay et al., 1997). After transformation in barley, this technology was quickly accepted as a preferred method. Biolistic and *Agrobacterium*-based transformation methods have been compared in barley (Travella et al., 2005; Harwood, 2012).

Advantages of *Agrobacterium*-based transformation are integration of a gene into a plant genome with fewer copies, integration of certain parts of DNA in a stable manner, less rearrangement of transgenes, and expression stability compared to generations (Haliloglu & Baenziger, 2003). *The ability* to obtain shoots from single totipotent cell effectively is indispensable for successful genetic transformation in plants. Against dicotyledonous plants, it is difficult to regenerate plant from leaf tissue in cereals. It has been determined that other gene transfer target explants such as immature embryos (Shrawat & Lorz., 2006), embryogenic pollen cultures (Kumlehn., 2006), isolated ovaries (Holme et al., 2006) and mature embryos are useful in cereals from which, mature embryos are germinated and regenerated easily and reliably, and become an ideal system for the production of barley (Hensel et al., 2009; Rostami et al., 2013). While there is a need for high transformation efficiency to minimize the effort required to produce sufficient number of independent transgenic plants for analysis, there are many other desirable properties of transformation systems including simplicity and low cost, desirable for providing maximum access to this technology.

Desired expression levels of transgenes and transgenic expression models are important (Harwood, 2012).

A reporter gene codes an enzyme with an easily screenable activity that is used to report the transcription activity of a gene of interest.With the recombinant DNA methods, the original promoter of the reporter gene is removed and replaced with the promoter of the investigated gene. To determine expression patterns of environmental or developmentally regulated genes, reporter genes are placed under the transcriptional regulation promoters that exhibit relevant developmental and/or stress responses (Karcher, 2002). In this study, we re-evaluated and optimized some factors related to the improvement of T-DNA transfer potential in barley. These factors are related to *in vitro* regeneration, explant treatment and co-cultivation duration.

Materials and Methods

Seed material

In this study, *Özen* genotype of barley was used as plant material. Seeds were obtained from the central research institute of field crops, Ankara, Turkey.

Regeneration

Seed sterilization

For surface sterilization of the barley seeds, intact grains were selected and the lowest disinfectant dose to which the highest success achieved, was determined as follows:

Solutions of 10, 20, 30, 40, 50, 60 and 70% doses of NaOCl were prepared from %5 sodium hypochlorite followed by immersion of the seeds in mentioned solutions for 15 minutes. Treated seeds were rinsed three times with sterile distilled water for 5 minutes each time. After the surface sterilization, seeds were subjected to the germination test on MS medium and % 20 dose was chosen in terms of the highest germination achieved (Data not shown).

Pre-treatment and regeneration

An experiment was conducted based on a completely randomized design in which MS media (Murashige & Skoog., 1962) containing vitamins, cytokinins and auxins with different ratios and combinations for shoot and root regeneration were prepared and 3% sucrose and 0.65% agar were mixed. Sterilized seeds were pre-treated for 24 hours in 4 different nutrient media (MS, MS+20 mg/l IBA, MS+20 mg/l BAP, MS+20 mg/l IBA+20 mg/l BAP) in sterile petri dishes. All cultures were kept at 24±1°C in 16 hours light photoperiod under white fluorescent light. Then, germinated seed explants (embryos) transferred to MS medium containing cytokinins and auxins using 7 different ratios and combinations prepared for shoot regeneration at high rates, suitable for gene transfer. Hormones of 0.1 mg/l NAA+(0.15 mg/l BAP, 0.30 mg/l BAP, 0.45 mg/l BAP, 0.60 mg/l BAP, 0.75 mg/l BAP, 0.90 mg/l BAP and 1.05 mg/l BAP) were used in the regeneration media preparation.

GUS gene transmission

Bacterial material

Agrobacterium tumefaciens GV 2260 strain as the host of the binary GUS-INT vector (Vancanneyt et al., 1990) for genetic transformation was obtained from the field crops department, faculty of agriculture, Ankara University, Ankara, Turkey.

Agrobacterium tumefaciens preparation and inoculation

One loop of *A. tumefaciens* GV 2260; 35 GUS-INT strain was obtained from the culture stored at -85˚C and inoculated with 10 ml of broth nutrient solution (25 mg/l Rifampicin and 50 mg/l Kanamycin), followed by shaking at 180 rpm and 28°C overnight to log phase (OD 600 ≅ 0.8) and following proliferation of liquid bacterial cultures were treated for 30 minutes with isolated embryo explants from MS medium germinated seeds (embryo explants that were placed at +4°C and subjected to cold treatment for 24 hours and embryo explants that were removed just before inoculation) followed by adding acetosyringone (inoculated with *A. tumefaciens* strain GV 2260) in a sterile cabin. Embryos were cultured in co-cultivation (MS) medium at 24±2°C for 24 and 48 hours in growth chamber with 16 hours light photoperiod with Grolux fluorescent light (40 µmol/m^2/s) (Figure 1). Embryos were then removed from the co-cultivation medium and transferred to a selection medium containing 400 mg/l Duocid and 75 mg/l Kanamycin (Duchefa, Holland) and were cultured. In this environment, developing and regenerating shoots were kept under continuous control and the expression (symptoms) of the transferred genes was observed (Figure 2). Two weeks after culture, percent of green plants was determined and then, planted in sterile peat soil by removing from culture vessels and rinsing with tap water (Figure 3). Each plant was covered with transparent coating and transferred to the growth chamber at 20-24°C for two weeks to acclimatize and allowed to grow there. Two weeks later, plants were transferred to the soil-filled pot with peat around their roots and then, plants were transferred to the greenhouse to develop (Figure 4).

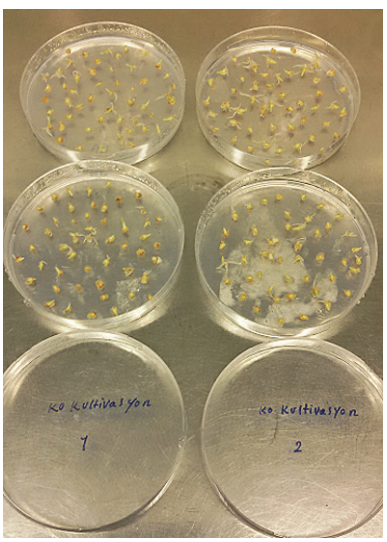

Fig. 1. Transfer of explants to the co-cultivation environment

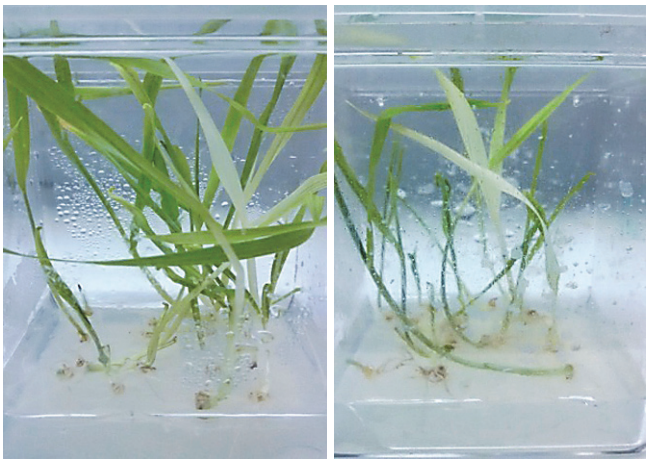

Fig. 2. Observation of the plants growing in the selection environment

Fig. 3. Planted green plants in sterile peat soil

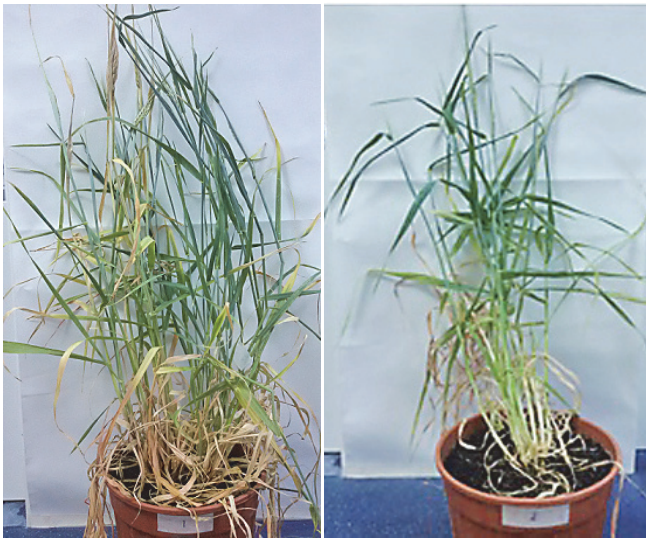

Fig. 4. Transfer of plants to soil-filled pots

Histochemical GUS assay

The leaves of the putative transgenic plants were taken to the histochemical GUS assays according to Khawar et al. (2004). Leaf samples were placed in 1 mM X-GLUC (5-bromo-4-chloro-3-indole) β-D glucrunic acid, 50 mM sodium phosphate, pH 7.0, 0.1% Triton X-100 and incubated for 24 hours at 38°C. Tissues were then rinsed occasionally with 96% ethanol during 2 days to digest chlorophyll content. Presence of GUS enzyme activity determined by blue staining in tissues (Figure 5).

PCR analysis

DNA was isolated from the leaves of the developed and putative transformed plants (Figure 6). Leaf samples were frozen in liquid nitrogen before crushing. Standard PCR techniques were used to identify the *nptII* sequence in selected leaves and seed samples. *nptII* primers having F-TTG CTC CTG CCG AGA AAG, R-GAA GGC GAT AGA AGG CGA primers sequences were tested (Figures 7 and 8).

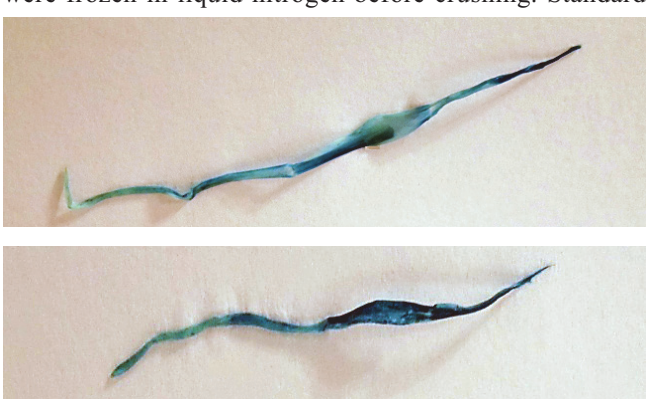

Fig. 5. Example of GUS positive leaf samples as a result of histochemical GUS assay

PCR reaction conditions

3 µl DNA, 5 µl 5X PCR tampon, 3 µl $MgCl_2$ (25 mM), 3 µl dNTP (10 mM), Primer F 4 µl, Primer R 4 µl, 0.3 µl Tag DNA polymerase, 2.7 µl dH_2O, made in 25 µl total volume.

Reactions were performed under 68°C touchdown PCR cycle conditions in a Biometra Tpersonal Thermocycler instrument as follows:

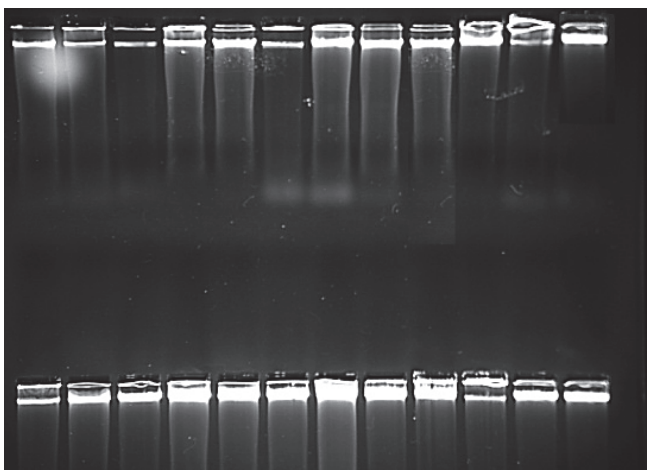

Fig. 6. Isolation of DNA from leaves of putative transformed plants

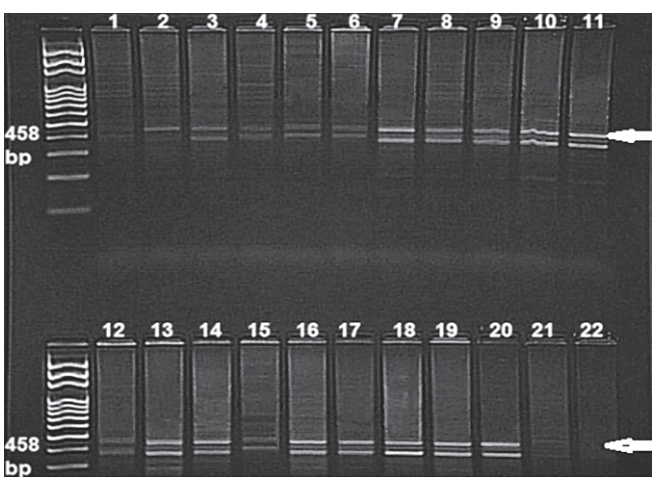

Fig. 7. PCR results of putative transformed plants

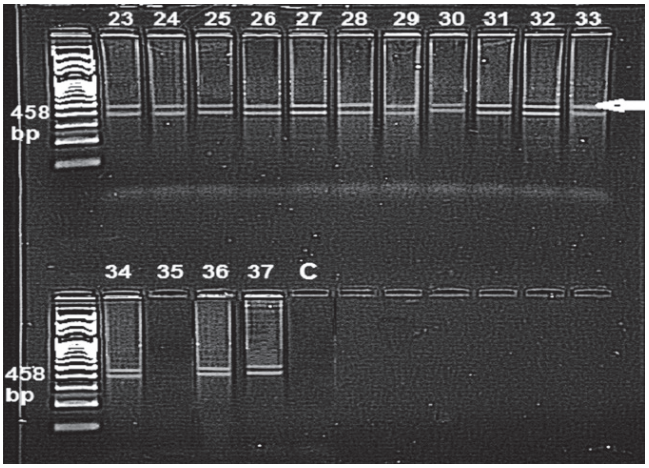

Fig. 8. PCR results of putative transformed plants

1) 94°C for 3:00 min, 2) 94°C for 1:00 min, 3) 68°C for 1:45 min, -1°C per cycle, 4) 72°C for 2:00 min 5) GO TO step 2. 9X, 6) 72°C for 2:00 min, 7) 94°C for 1:00 min, 8) 59°C for 1:45 minute, 9) 72°C for 2:00 min, 10) GO TO step 7. 19X, 11) 72°C for 10:00 min, 12) 10°C for ∞ (Figures 7 and 8).

Observations and statistical analysis

16 replicates containing 5 explants each (total sum of 16×5 = 80 explants) were applied. Data were subjected to variance analysis using MSTAT-C statistical program and mean comparison tests were carried out with Duncan's multiple range test.

Results

***In vitro* plant regeneration**

After plants were regenerated and improved on regeneration medium for 2 weeks, they were removed from media and different growth features including germination percent, number of shoot per explant, root formation percent, number of root per explant, root length and leaf area were measured. According to the results, priming factor was significant ($P < 0.01$) for all of the measured traits except for root number per explant.

Hormone factor was significant ($P < 0.01$) for germination percent, root formation percent and root length, and for number of shoot and root per explant ($P < 0.05$). Interactions were sigificant for all of the characteristics studied ($P < 0.01$) except for number of shoot per explants ($P < 0.05$). According to the mean comparisons, nutrient medium containing 20 mg/l BAP was determined as the best medium among the priming media having highest level of germination percent (100), root formation percent (99.05), number of root per explant (4.14), root length (4.05) and leaf area (5.14) (Table 1).

In regeneration medium, germination percent was significantly affected by culture medium ($P < 0.01$) so that, 0.30, 0.45 and 0.60 levels caused the highest germination percent of 100% for all three media. Also, culture medium was significant ($P < 0.05$) for number of shoot per explant and the highest values were obtained from 0.3 level of BAP (1.044). Root formation percent significantly ($P < 0.01$) was affected by culture medium and the highest value (99.10) was gained from 0.45 mg/l BAP in culture medium. Culture medium was also significant ($P < 0.05$) for number of root per explant and the highest value of this trait (4.443) was obtained using 0.15 mg/l BAP in culture medium. Finally, root length was significantly ($P < 0.01$) affected by culture medium and 0.45 mg/l BAP caused the highest value of 4.22. In general, nutrient medium containing 0.1 mg/l NAA+0.45 mg/l BAP at the hormone level, was observed as the best one having the high amount of number of shoot per explant (1.033), number of root per explant (4.178), leaf area (4.424) and the highest value of germination percent (100), root formation percent (99.10) and root length (4.229) (Table 2).

Genetic transformation

PCR reactions were separated by 1.5% agarose gel electrophoresis. Electrophoresis performed in 70 volts for

Table 1. Mean comparisons of different priming media on germination percent, number of shoot per explant, percent of root formation, number of root per explant, root length and leaf area

Priming	Germination percent		Number of shoot per explants	Root formation percent	Number of root per explants	Root length, cm	Leaf area, cm^2
MS (Control)	98.93 b*	1.06 a	96.66 ab	3.75 a	3.02 b	4.53 b	
MS+20 mg/l IBA	98.81 b	1.00 b	92.06 c	3.97 a	3.79 a	3.95 bc	
MS+20 mg/l BAP	100.0 a	1.013 b	99.05 a	4.14a	4.05 a	5.14 a	
MS+20 mg/l IBA+20 mg/l BAP	98.98b	1.00 b	95.48 b	3.76a	3.27b	3.40 c	

*Data with the same letter are not significantly different using Duncan's multiple range test at $P < 0.05$.

Table 2. Mean comparisons of different plant growth regulatory media on germination percent, number of shootper explant, root formation percent, number of root per explant, root length and leaf area

Culture medium 0.1 NAA (mg/l) + BAP (mg/l)	Germination percent	Number of shoot per explants	Root formation percent	Number of root per explants	Root length, cm	Leaf area, cm^2
0.15	97.92 c*	1.000 c	93.35 b	4.443 a	3.302 cd	4.097 a
0.30	100.0 a	1.044 a	96.94 ab	3.993 abc	3.599 bc	4.437 a
0.45	100.0 a	1.033 ab	99.10 a	4.178ab	4.229 a	4.424 a
0.60	100.0 a	1.008 bc	97.49 ab	3.967abc	3.919 ab	4.192 a
0.75	99.10 b	1.036ab	93.54 b	3.623bc	2.859d	3.819 a
0.90	99.11b	1.014 bc	96.92ab	3.63bc	3.538bc	4.442 a
1.05	98.12 c	1.008 bc	93.33b	3.53c	3.312cd	4.385 a

* Data with the same letter are not significantly different using Duncan's multiple range test at $P < 0.05$.

1 hour and after electrophoresis termination, the gel was checked on UV lamp. Results showed that cold pretreatment (+4°C) prior to *Agrobacterium*-infection along with longer co-cultivation duration resulted in improved transformation efficiency when cold treatment and 48 hours co-cultivation time applied. The addition of a cold treatment appeared to have synergistic effect on infection and transformation efficiencies.

Percent of the green and survived plants obtained from the selection medium, percent of plant with positive PCR and percent of plant with positive GUS showed higher values in embryos treated at +4°C for 24 hours than those of control (non-treated) embryos for both 24 and 48 hours of co-cultivation period (Table 3). The highest rate was obtained in percent of green plants (17.18), percent of plants with positive GUS (15.62) and percent of plants with positive PCR (14.06) in explants that were subjected to cold treatment for 24 hours and then co-cultivated for 48 hours and the gene transfer frequency was higher (Table 3) and the transformation ratio was at least 4.5 times greater than that of control condition (without cold treatment) and 48 hours explants co-cultivation duration.

Prolonging the cultivation period from 24 to 48 hours in control conditions reduced the rate of green survived plants, plants with positive PCR and plants with positive GUS transformation in normal conditions (without explants cold treatment). This indicates the interaction between these two factors and with cold pretreatment of the explants, plant response changed against increase in the duration of the co-cultivation period. This can be due to the decrease in the production of ROS compounds such as H_2O_2 and consequently, reduce in tissue damage. Also it has been cleared that cold treatment reduces plant defense system against bacterial invasion (Zhang et al., 2013). GUS analysis of leaves taken from developed plants in the glasshouse showed GUS activity in transgenic plant leaves irrespective of their place and position on the plant. Seeds of the transgenic plants collected and (T1) plants were tested for GUS expression and proved GUS positive in Mendelian model 9:3:3:1.

In this study, rapid, efficient and reproducible *in vitro* regeneration and *Agrobacterium*-based transformation system were developed with mature embryo explants for barley and wheat.

Discussion

Plant engineering is a powerful technique for direct development of commercial plants and developing new plants that express a number of valuable characters. High regeneration capacity is a fundamental requirement for the construction of an effective genetic transformation protocol. Three areas are taken into consideration for the improvement of transformation efficiency. The first is improvement of regeneration in the target tissues, the second is improvement of the number of transformation events, and the third is improvement of the selection of transformants. The first requirement key for a successful transformation system is that tissues can be highly regenerated (Harwood, 2012). Although the target tissues mostly are immature embryos, but microspore (Shim

Table 3. GUS gene transfer frequency table with different explants treatment and different co-cultivation period

Explant treatment	Cold treatment	Control (non-treated)	Cold treatment	Control (non-treated)
Co-cultivation time (hour)	24	24	48	48
Green plant percent	15.17	8.75	17.18	3.12
Percent of plants with +GUS (%)	8.92	3.75	15.62	3.12
Percent of plant with +PCR (%)	9.82	6.25	14.06	3.12

et al., 2009) and ovule (Holme et al., 2008) have been used, but mature embryos and leaf-based apical meristems have advantages over immature tissues which are easier to use and are obtained in large quantities throughout the year without any problem of seasonal effects on regeneration (Sharma et al., 2005a; Rostami et al., 2013).

In our study, results of the hormone pretreatment are in accordance with the results found by Dissanayaka et al. (2015) showing that pre-sowing of *Exacum trinervium* seeds with 6-Benzylaminopurine (BAP) resulted in enhanced seed germination and seedling vigour and growth parameters of *E. trinervium* were significantly affected by BAP compared to control. Therefore, the present study suggests that BAP treatment may be involved metabolically in stimulation of germination and hence, and in increment of the seedling vigour of barley. Also, it has been found that *in vitro* germination and regeneration of hybrid banana embryos was affected by hydro and hormonal priming so that hydro-priming resulted in %40.8 germination compared to %20.8 germination of non-primed seeds, and priming in GA3 for three days enhanced germination two fold and also enhanced growth parameters in this plant (Arun et al., 2013).

In cereals which are not typically good hosts for *Agrobacterium*, deviations from optimal conditions are very difficult to tolerate. The most critical variables affecting gene transfer during co-cultivation are nutrient concentration, temperature, presence and concentration of acetosyringone, and its duration. It has been suggested that these factors co-act at the beginning of the transformations that induce the expression of *Vir* genes (He et al., 2010; Manfroi et al., 2015).

Results of our research of cold pretreatment of explants before *Agrobacteium*-infection, is in accordance with the results obtained by the Zhang et al. (2013) which reported improved transformation efficiency to about 20% in rice and rye explants that exposed to cold shock pretreatment prior to *Agrobacteium*-infection. Also, cold shock treatments facilitated root fragment transformation and crown gall formation in *Arabidopsis* (Zhang et al., 2013). Furthermore, it has been known that the cold treatment greatly reduced callus browning after infection and these can be cited as the reasons for the increase in the events of the transformation.

Co-cultivation duration is another important factor affecting the transformation efficiency. While the optimum co-cultivation duration of barley was one-day in control condition, the optimum period changed to two days with the cold pre-treatment of the explants and also, the amount of transformed plants was remarkably increased. This indicates that the cold application not only increases the amount of genetic changes on first day, but the increase is higher on second day. In a research on *Agrobacterium*-infection of soybean half-seed explants after 3, 4, 5, and 6 days of co-cultivation, it was revealed that 5-day co-cultivation was the most suitable, with strong vitality of the explants (Li et al., 2017).

Also, in another research for optimizing of the pre-culture and co-cultivation durations, different pre-culture and co-cultivation durations were tested and it was found that direct infection without pre-culturing and with a 2-day co-cultivation period from 0, 1, 2, and 4 days co-cultivation caused maximum GUS expression (Zhang et al., 2017). In other research performed by Li et al. (2017) to evaluate the factors affecting transformation frequency of *nisqually-1* as the first sequenced model tree, it was shown that co-cultivation time clearly affected transformation efficiency and 2-day co-cultivation resulted in the highest frequency of the Gus-positive shoots.

It has been revealed that prolonged time of co-cultivation increased the transformation frequency and subsequent increase in co-cultivation time decreased this trait which caused over growth of bacteria and the bacterial leaching in longer co-cultivation periods resulted in negative effects (Yadav et al., 2012; Zhang et al., 2017).

In this study, rapid, efficient and reproducible *in vitro* regeneration and *Agrobacterium*-based transformation system were developed with mature embryo explants for barley and could be used in other similar monocots.

Conclusion

A protocol for tissue culture regeneration of zygotic embryo was developed. Certain changes, along with the changes in the explants subjection to the cold treatment resulted in increased transgenic ratio. Pre-treatment of the seeds with BAP cytokinin hormone prior to explant production, led to the favorable result in explant regeneration increment in regeneration medium. We succeeded to increase regeneration rate in all evaluated traits than control. Pre-treatment of 20 mg/l BAP along with 0.45 mg/l BAP in regeneration media resulted in the highest germination percent (100%), root formation percent (100%), root number per explant (5), root length (4.86 cm), and leaf area (6.04 cm^2). On the other hand, explants treatments with cold in transformation trials illustrated new but opposite results in different co-cultivation levels compared to control so that, cold treatment not only improved regeneration efficiency than control, but remarkably caused the changes in response trend to increase co-cultivation period and transformation efficiency was significantly increased with increase in co-cultivation period. The method can be used for transformation of barley with other strains *of A. tumefaciens* for development of transgenic plants with improving stress tolerances.

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Received: June, 1, 2018; *Accepted:* July, 5, 2019; *Published:* December, 31, 2019