Research Article

The Effect of Endogenous Hormones, Total Antioxidant and Total Phenol Changes on Regeneration of Barley Cultivars

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Background: Barley (*Hordeum vulgar* L.) is a valuable platform for producing recombinant proteins. Before using different barley cultivars as an efficient platform for molecular farming, optimization of cultural conditions and studying the effective factors on the tissue culture are critical.

Objectives: In this study, we evaluated callus induction, plant regeneration and changes in the levels of total antioxidant, total phenol and endogenous hormones of three Iranian barley cultivars (Reyhan, Yousef and Bahman) and Golden Promise cultivar.

Materials and Methods: We used immature embryos as explants on MS-based medium containing 3 mg.L^{-1} 2,4-D for callus induction. Calluses were transferred to regeneration media with 2 mg.L^{-1} BAP. The levels of endogenous hormones were measured using High-Performance Liquid Chromatography system and total antioxidant and total phenols were determined using a spectrophotometer.

Results: We demonstrated that callus formation was very high in all cultivars (about 91%) and all immature embryo explants had the potential to produce embryogenic calluses. The present study also showed that the regeneration rates among the studied cultivars were very different and the Iranian cultivars showed lower regeneration percentages (about 1.4%) compared to Golden Promise cultivar (about 72.5%). The levels of endogenous hormones in Iranian cultivars and Golden Promise varied distinctly and significant differences in terms of total antioxidants and total phenols were found in the two groups.

Conclusions: Accumulated evidence suggests that for successful regeneration of recalcitrant cultivars, external treatments should be done in a way to reduce the inhibitory effects of internal factors.

Keywords: Antioxidants, Barley, Callus induction, Endogenous hormones, Phenolic compounds, Regeneration

1. Background

Barley, one of the species of the tribe *Triticeae* in the *Poaceae* family, is known as one of the most important crops throughout the world. Barley is also a valuable platform for producing recombinant proteins (1). In order to use barley as molecular farming platform, a simple and speedy regeneration method is very important. In addition, the callus induction and regeneration of barley are highly genotype dependent and finding desired genotypes is one of the important goals of molecular farming. Among barley cultivars, Golden Promise and Igri are known as the most reliable and efficient cultivars for transformation (2). Endogenous hormones, antioxidants, and phenolic compounds are the most important factors affecting plant tissue culture and regeneration (3). Different hormone compositions are used for embryogenic callus formation and subsequent shoot regeneration. It suggested that the abilities of embryogenesis and regeneration for the callus derived from immature embryo may be dependent on not only hormone composition in the medium, but also on the hormone level in the callus. Success in tissue culture depends on the factors that favor the formation of friable and high quality callus in a shorter time competent for shoot regeneration. Detection of correlation between variations of endogenous hormone levels and plant regeneration is critical for the *in vitro* regeneration of non-regeneration barley cultivars (4). The relationships

between endogenous hormone contents and somatic embryogenesis have been the subject of many studies (5, 6).

In addition to endogenous hormones, phenolic compounds and antioxidants may also affect the success of *in vitro* cultures (3). *Reactive oxygen species* (*ROS*) interact with plant hormones and affect callus induction directly or indirectly (7). Based on the inverse relationship between ROS and antioxidants, antioxidant measuring is a reliable method for detecting the level of ROS (8). The oxidized products would be able to prevent explant development and cause the death of the explants (9). The phenolic compounds are also effective in tissue culture especially plant regeneration (10). The heterogeneity of natural phenols and the possibility of interference with other oxidizable compounds, especially sugars, led to the design of various methods to assay the total amount of phenolic compounds (11). It was also reported that various 1-naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP) concentrations significantly affect phenolic exudation (12)

2. Objectives

Here we analyzed the regeneration ability and levels of endogenous hormones, antioxidants, and phenols in embryogenic calluses derived from immature embryos of barley. We also compared the antioxidants, phenols, and hormone profiles and tissue culture responses of immature embryo in the barley cultivars.

3. Materials and Methods

3.1. Plant Materials

Seeds of barley cultivars (Bahman, Yusef, Reyhan, and Golden Promise) used in this study were kindly provided by the Department of Cereal Research at the Seed and Plant Improvement Research Institute (SPII), Karaj, Iran. Golden Promise was used as a model genotype to compare with Iranian cultivars. All seeds were potted to a depth of 5-10 cm in a soil mix of compost: Perlite: Grit (2:2:1). The pots were placed in temperature-controlled glass rooms at 15 °C under natural light with a relative humidity of 60%. After 12 weeks, spikes with 1.5-2 mm embryo length were harvested and used for experiments.

3.2. Isolation of Barley Immature Embryos

The immature seeds harvested from main spikes were surface sterilized with ethanol 70% for 30 seconds, sodium hypochlorite 5% for 12 minutes and washed several times with sterilized distilled water. Immature embryos were then excised from seeds under the binocular.

3.3. Callus Induction

The scutellum part of immature embryos was placed downward on Murashige and Skoog medium (MS) containing B5 vitamins, 3% (w.v) maltose, 0.4% (w.v) phytagel. The MS medium was supplemented with 3 mg.L^{-1} 2,4-D. The experiment included five independent replications with 20 explants in each replication. The subculture was done in 2 weeks intervals. The cultures were incubated in darkness for 6-8 weeks.

3.4. Shoot Regeneration

After 6-8 weeks, the embryogenic calluses were transferred to shoot regeneration medium containing MS basal salts, B5 vitamins, 2% (w.v) maltose, 0.4% (w.v) phytagel, and 2 mg.L^{-1} BAP. Regenerated shoots were rooted on hormone-free MS medium. Regeneration and rooting were done at 25°C under a 16/8-h (day/night) photoperiod provided by cool-white fluorescent lamps at an intensity of 30 mmol photons/m^{-2} s^{-1}.

3.5. Analysis of Endogenous Hormones

Extraction and quantification of indole acetic acid (IAA), zeatin (ZT), gibberellic acid (GA3), and abscisic acid (ABA) from calluses were conducted as described by Ji-Hye Seo et al. and Peter J. Davies (13, 14) with modifications. Callus samples of 1 gr powdered in liquid nitrogen were dissolved in ethanol 80% and placed on a shaker for 4 hours at 200 rpm. The extract was centrifuged at 4 °C and 4000 g for 20 minutes and then the supernatant was transferred to a new 15 mL centrifuge tube. The extract was stored at 4 °C for the next experiments Ethanolic extract was dried under vacuum in a rotary evaporator. The dried extract was dissolved in 10 ml methanol and used for reverse-phase HPLC.

For HPLC analysis of callus extracts, a mixture of methanol and water was used as the mobile phase. Based on the reported compositions of the mobile phase, gradient elution mode (0-23 min, 25%-75% methanol) was used. Detection was carried out at 208, 254, 265, and 280 nm by a UV-Visible detector for GA3, IAA, ABA, ZT, respectively. Quantitative determination of endogenous hormones was carried out by comparing the peak area of the samples with the corresponding peak areas of standard solutions containing known concentrations of GA3, IAA ABA, and ZT.

3.6. Measurement of Total Antioxidant

In this experiment, methanolic extracts of embryogenic calluses were used to determine their antioxidant activity using the ABTS$^+$ test (15). To prepare ABTS$^+$ cations, 10 mL of water solution 7mM ABTS and 10 mL of water

solution 2.45 mM potassium persulfate, were mixed in the ratio 1:1 and stored in darkness at 4 °C for 16 hours. This method produced blue-green radicals. ABTS⁺ cation solution was diluted by phosphate buffer 50 times. The base of the dilution is the absorption of 0.75 ± 0.05 at 734 nm. ABTS⁺ cation solution had been absorbed in 415, 645, 734, and 815 nm but at 734 nm showed the highest absorption. Thirty μL of the methanolic extracts of embryogenic calluses was added to 2 mL of diluted ABTS⁺ solution and the absorption at 734 was measured every minute after initial mixing. Data were analyzed in a Completely Randomized Design (CRD) with three repetitions. The inhibition of radicals was calculated using the following formula:

Scavenging Effect (%) = ((Absorbance control - Absorbance sample)/Absorbance control) * 100

3.7. Measurement of Phenolic Content by Folin-Ciocaltu Method

The total phenolic concentration in the extracts was determined using the Folin-Ciocalteu reagent according to the methodology suggested by García-Perez *et al.* (2012), using gallic acid as a standard (16). To prepare FC reagent, 10 gr of sodium tungstate and 2.5 gr of sodium molybdate were dissolved in 70 mL distilled water. Ten mL of concentrated hydrochloric acid and 5 mL of 85% phosphoric acid were added and the mixture

was heated for 10 hours and ultimately, it was cooled at room temperature. 15 gr lithium sulfate was then added and final volume brought to 100 mL with distilled water. Ten μL of FC reagent was mixed with 60 μl of the methanolic extracts of embryogenic calluses then 1700 μL of distilled water was added. After 3-8 minutes in darkness, 300 μL of 20% sodium carbonate was added and the mixture was then kept in darkness for 2 hours. The absorption of each sample was measured at 740 nm. Absorptions of concentrations 0.6, 1.2, 1.8, 2.4, 3.5, and 5 mg.L^{-1} of gallic acid were measured and the standard curve was produced.

3.8. Statistical Analysis

Data were analyzed with R (version: 8.01) and Libreoffice calc (Version: 6.3.3.2) software (17). Tukey's post hoc test was used to assess statistical significance among treatments.

4. Results

4.1. Callus Induction and Shoot Regeneration

All 4 cultivars used in this study were able to produce embryogenic calluses. It was also observed that Golden Promise was more responsive to shoot regeneration (about 72.5%) when compared to the 3 Iranian cultivars, ranging between 0.75% and 2.5% (**Fig. 1A**), which
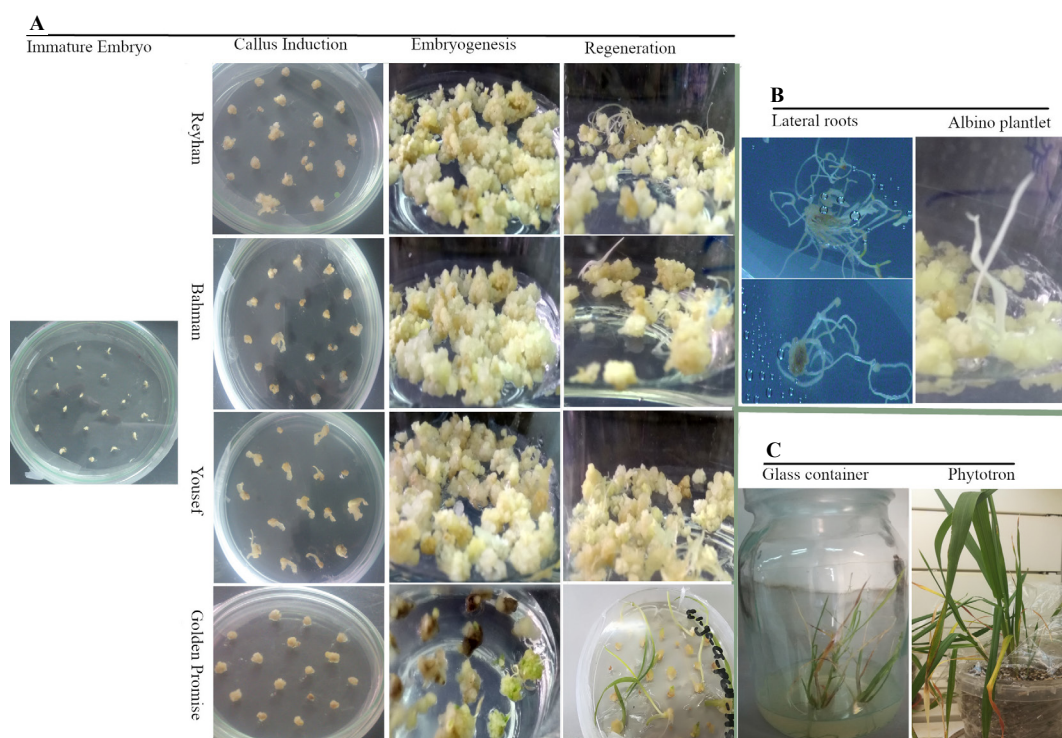

Figure 1. The *in vitro* tissue culture of Golden Promise and 3 Iranian barley cultivars (A) immature embryo cultures for regeneration of Golden Promise and Iranian barley cultivars, (B) production of lateral roots and albino plantlets on calluses of Iranian barley cultivars, (C) regeneration of Golden Promise cultivar.

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clearly shows that there exists variation among different cultivars in their tissue culture response (**Fig. 2A**). Statistical analysis showed significant differences ($P \leq 0.01$) among the studied cultivars (**Table S1**). Unlike the significant difference between the Golden Promise and Iranian cultivars, there was no significant difference among the Iranian cultivars (**Fig. 2B**). Some calluses of the Iranian cultivars produced albino plantlets during the regeneration stage (about 2%) while, some of them only produced many lateral roots (about 6.2%) (**Fig. 1B**).

4.2. HPLC Analysis of Callus Extracts
Under the chromatographic conditions described in 3.5, retention times were: 12.149, 12.679, 18.210, and 19.879 minutes for standard samples: GA3, ZT, IAA, and ABA, respectively (**Fig. 3**).

Our results showed that ABA level in Golden Promise was about 7 times lower than Iranian cultivars (**Fig. 4A**). Statistical analysis revealed a significant difference ($P \leq 0.01$) among the examined barley (**Table S1**). Post hoc test showed that there was a significant difference between Golden Promise and the other cultivars but among the 3 Iranian cultivars, there was no significant difference (**Fig. 5A**).

IAA concentration was higher in Iranian cultivars than Golden Promise (**Fig. 4B**). Statistical analysis showed that there was a significant difference ($P \leq 0.01$) between the cultivars of barley (**Table S1**). Based on the post hoc test, there was no significant difference among Iranian cultivars but the difference between Iranian cultivars and Golden Promise was significant (**Fig. 5B**). The concentration of IAA in Iranian cultivars compared to Golden Promise was very high (**Fig. 4B**).

The HPLC results showed that the ZT level in the Iranian cultivars was much lower than the Golden Promise cultivar (**Fig. 4C**). Statistical analysis showed that there was a significant difference among cultivars (**Table S1**). Furthermore, based on the post hoc test, there was no significant difference among Iranian cultivars but the difference between Iranian cultivars and Golden Promise was significant (**Fig. 5C**).

HPLC results showed that the amount of GA3 was considerably high in Golden Promise (**Fig. 4D**). A significant difference was observed among all cultivars (**Table S1**). Based on post hoc test, it was found that in addition to the difference between Iranian cultivars and Golden Promise, there was a significant difference among Iranian cultivars (**Fig. 5D**).

4.3. Measurement of Total Antioxidant Activity
Antioxidant activity was observed in all cultivars. The highest levels of scavenging effect were obtained in callus extract of Golden Promise cultivar (53.3%) and
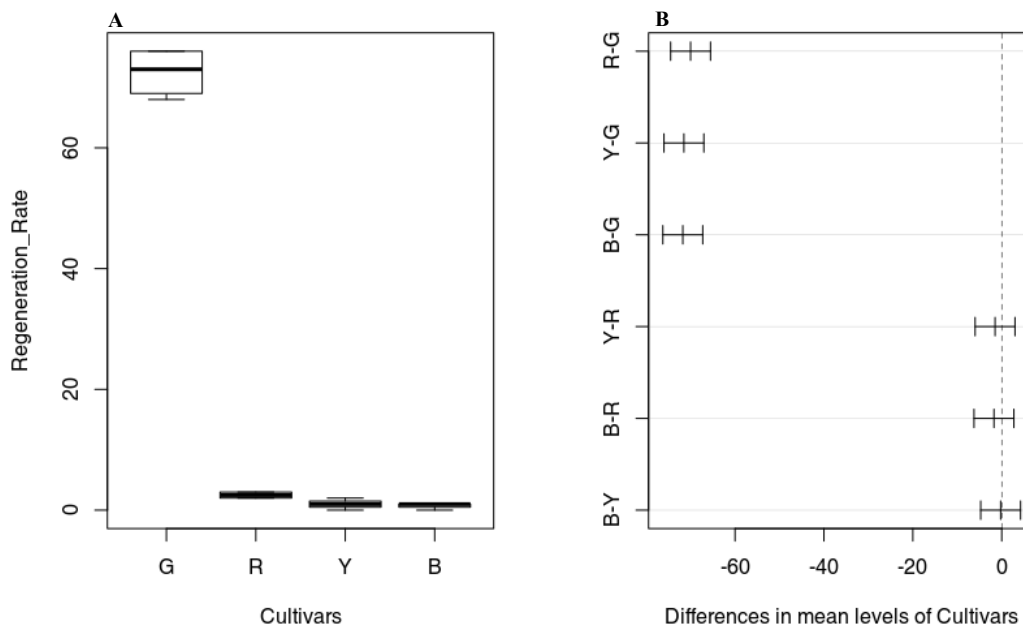

Figure 2. Analysis of regeneration rate of Golden Promise and 3 Iranian barley cultivars from embrogenic callus (A) Regeneration percentage of Golden Promise and 3 Iranian barley cultivars. (B) Comparison of means of barley cultivars in terms of regeneration rate. The data were analyzed by one-way ANOVA, and tested for significance using Tukey's HSD Post-Hoc test. For comparison across cultivars for regeneration rate, those that do not contain 0 are significantly different at $p \leq 0.01$. The letters used to represent the different cultivars examined are: R, Reyhan; B, Bahman; Y, Yousef, G, Golden Promise.
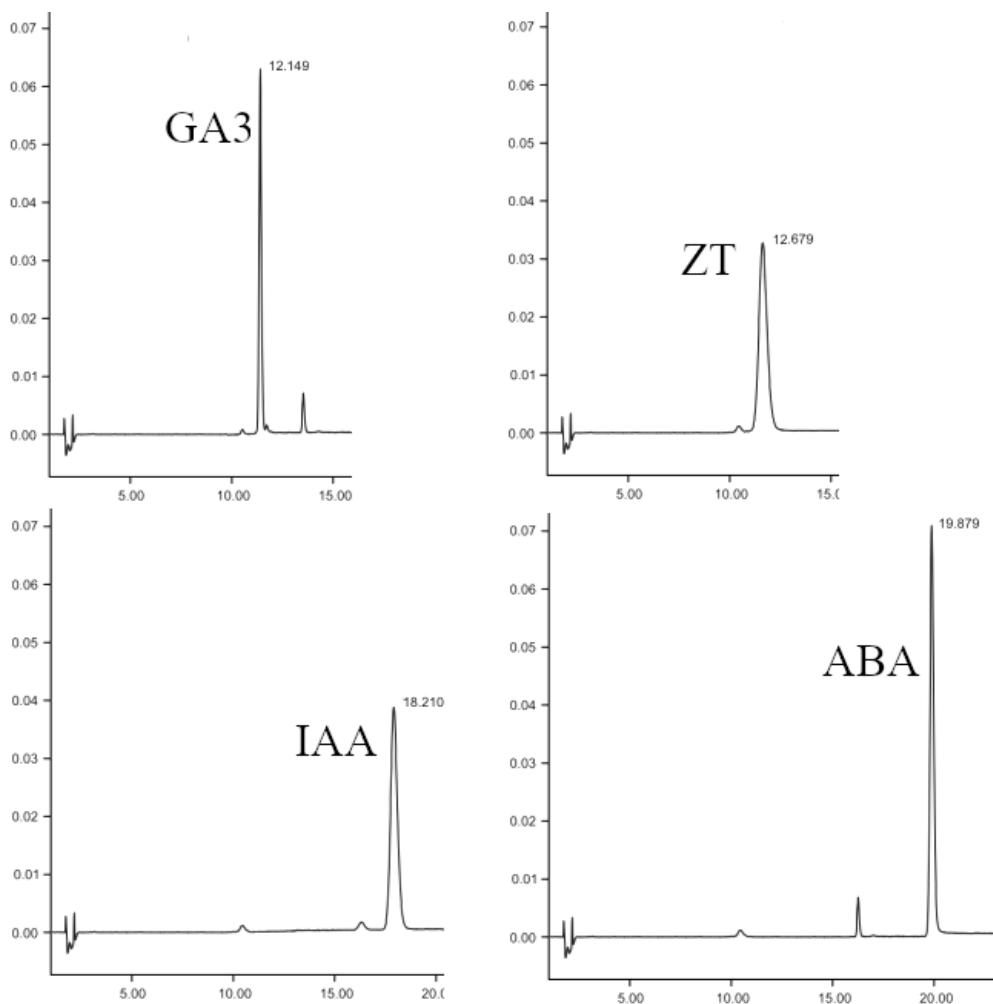
Figure 3. The retention times of standard samples gibberellic acid (GA3), zeatin (ZT), indole 3-acetic acid (IAA), and abscisic acid (ABA). A mixture of methanol and water by gradient elution mode (0-23 min, 25%-75% methanol) was used. Detection was carried out at 208, 254, 265, and 280 nm by UV-Visible detector for GA3IAA, ABA, and ZT, respectively.
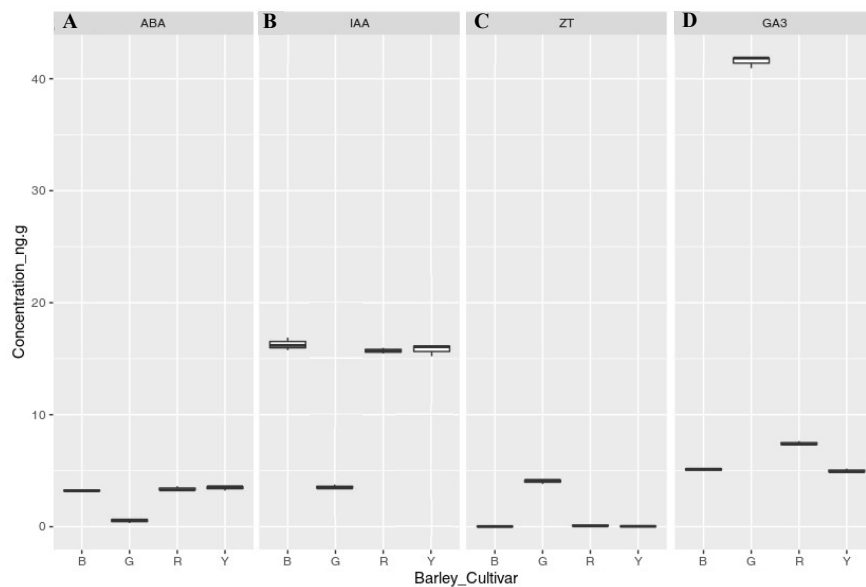

Figure 4. Comparison of endogenous hormone levels in the barley cultivars. (A) ABA; (B) IAA; (C) ZT; (D) GA3
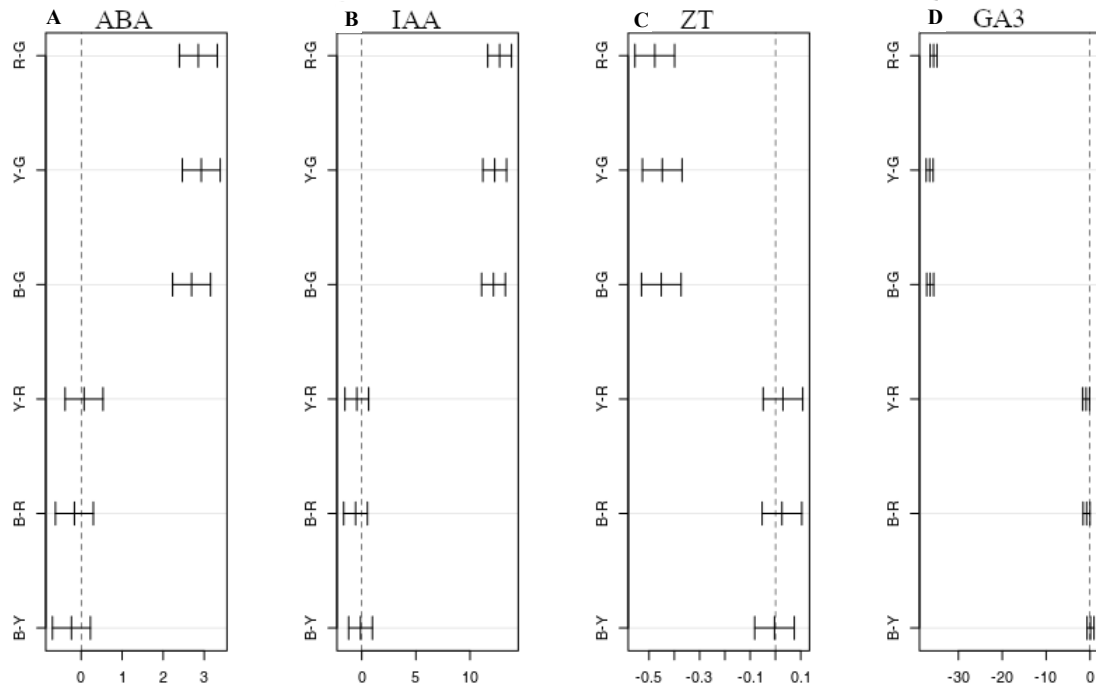
Figure 5. Difference of mean levels of endogenous hormone levels among the barley cultivars. The data were analyzed by one-way ANOVA, and tested for significance using Tukey's HSD Post-Hoc test. For comparisons across cultivars for Changes of endogenous hormone levels, those that do not contain 0 are significantly different at $p \leq 0.01$. (A) ABA; (B) IAA; (C) ZT; (D) GA3
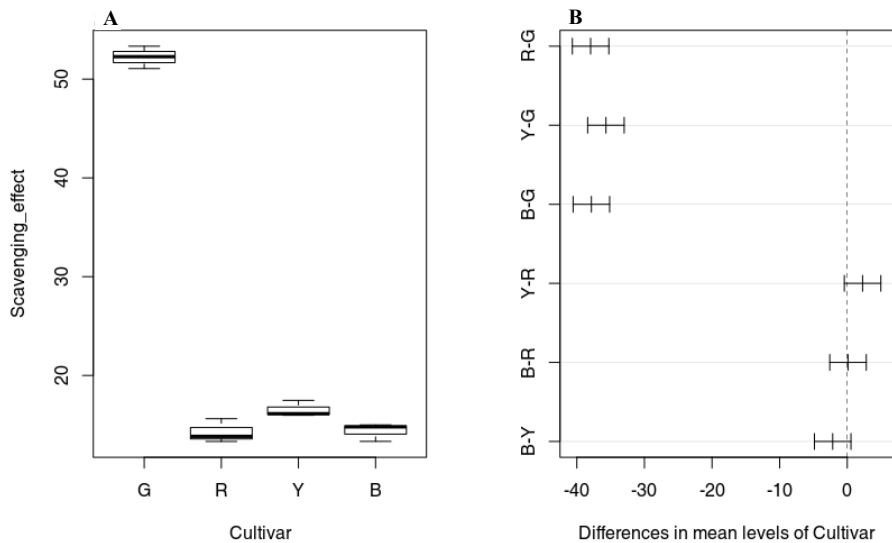

Figure 6. Measurement of Total Antioxidant Activity based on the scavenging effect of Golden Promise and 3 Iranian barley cultivars. (A) Scavenging effect of the barley cultivars; (B) Comparison of means of barley cultivars in terms of scavenging effect. The data were analyzed by one-way ANOVA, and tested for significance using Tukey's HSD Post-Hoc test. The letters used to represent the different cultivars examined are: R, Reyahn; B, Bahamn; Y, Yousef; G, Golden Promise

the 3 Iranian cultivars had lower scavenging effect compared with Golden Promise (**Fig. 6A**). Statistical analysis showed a notable difference ($P \leq 0.01$) between the Iranian barley cultivars (**Table S1**) and the Golden Promise but no difference was observed among the Iranian cultivars (**Fig. 6B**).

4.4. Total Phenolic Content of Embryogenic Calluses
An obvious variation in total phenolic content was observed among the 4 barley cultivars. The lowest amount was found in the Bahman cultivar (about 8 mg.g^{-1}) and the highest amount (about 20 mg.g^{-1}) was observed in Golden Promise cultivar (**Fig. 7A**). ANOVA
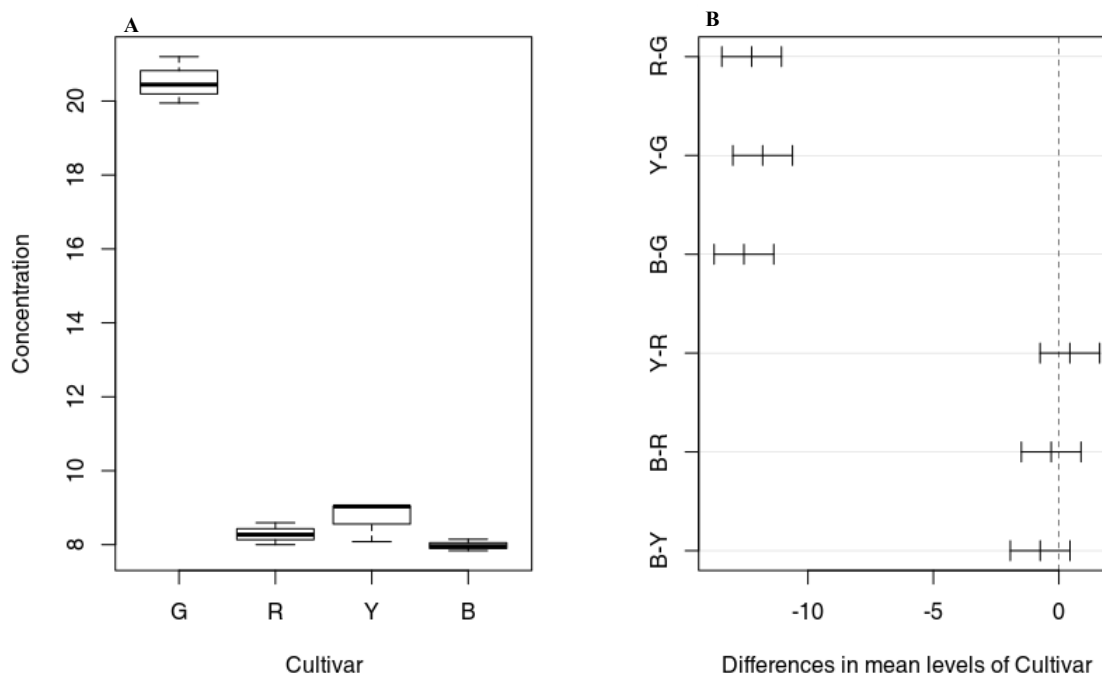
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Figure 7. Measurement of Total Phenolic Content of Golden Promise and 3 Iranian barley cultivars. . (A) Total Phenolic Content of the barley cultivars; (B) Comparison of means of barley cultivars in terms of Total Phenolic Content. The data were analyzed by one-way ANOVA, and tested for significance using Tukey's HSD Post-Hoc test.The letters used to represent the different cultivars examined are: R, Reyahn; B, Bahamn; Y, Yousef; G, Golden Promise

revealed a significant difference ($P \leq 0.01$) between the Iranian barley cultivars and the Golden Promise (**Table S1**); the amount in Golden Promise cultivar was very high, about three times more than the Iranian cultivars (**Fig. 7B**).

5. Discussion

Optimization of cultural conditions and identifying barriers for regeneration in commercial cultivars are of great value. Many studies have been carried out on regeneration and transformation of barley, in which different concentrations of growth hormones were used (18, 19). The formation of embryogenic callus in barley mainly depends on 2,4-D concentration, which 2-3 mg.L^{-1} of 2,4-D seems adequate in most cases (20). Our results showed that all cultivars of barley are able to produce embryogenic callus in MS medium containing 3 mg.L^{-1} 24-D. However, in the regeneration stage, Iranian cultivars showed a very low regeneration rate while it was very high in Golden Promise cultivar (**Fig. 1C**). Many articles have shown that regeneration capacity of wheat and barley varieties varied over a wide range of factors. This strong genotypic effect on regeneration has been observed in most graminaceous species (18, 19). Effect of genotype in regeneration has

been intensively studied and appreciated in not only barley but other cereal crops as well (21, 22).

ABA is connected with the vegetative development of plants by controlling gene expression (23). Torrizo and Zapata reported that in some varieties of rice, the concentration of ABA and regeneration have inverse relationships. Furthermore, they also showed that low ABA concentrations stimulated the proliferation of the callus (24). In a study on the regeneration of *Laelia anceps*, it has been revealed that higher ABA concentrations in the culture medium were associated with lower values regarding shoot height, number of leaves, and number of roots (25). The ABA has multiple physiological effects on plants in tissue culture; including stomatal occlusion and the inhibition of cell division and elongation, also has an antagonistic effect against cytokinin (26). We indicated that the Golden Promise cultivar, with a high regeneration rate, had lower ABA than the examined Iranian cultivars (with a low regeneration rate). The amount of ABA hormone in this specific cultivar was 7 times lower than local cultivars (**Fig. 4A**). Our findings confirm that the percentage of regeneration and the amount of ABA are inversely related.

In recent years, it has become apparent that plant

hormones rarely act alone. Plant developmental output is regulated by a complex network of interlocking hormonal signaling pathways. Plant regeneration patterns depend on the specific balance of endogenous hormones (27). There is antagonistic regulation between auxin and cytokinin. Genes contributing to the biosynthesis of auxin, suppress the cytokinin biosynthetic genes (28). A high auxin/cytokinin ratio induces root regeneration, whereas a low ratio promotes shoot induction (29). Direct interaction between auxin and cytokinin during shoot regeneration has recently been revealed using pistils as explants (30). In our study, cytokinin/auxin ratio in Golden Promise compared to Iranian barley cultivars was very high (about 333 times). So it can be said that high cytokinin/auxin ratio in Golden Promise enables a higher rate of regeneration compared to Iranian barley cultivars. In addition, it seems that high auxin/cytokinin ratio in Iranian cultivars caused the formation of lateral roots. Shoot meristem initiation requires spatially restricted distributions of both auxin and cytokinin in callus. Hisano *et al*. (2016) in their study on the effect of endogenous hormones on the regeneration of 2 malting barley and Golden Promise cultivars used the ls/ms/ms method. Considering malting barley cultivar had high regeneration rate, they concluded that the lower level of auxin in malting barley cultivars caused high regeneration rate. In the present study, we used embryogenic calluses of commercial cultivars with very high levels of auxin compared to Golden Promise. Therefore, high auxin/cytokinin ratio might lead to the low regeneration rates of Iranian barley cultivars and continuous lateral root formation on callus tissues. In addition Hisano *et al*. (2016) showed the levels of cytokinin in cultivars with high regeneration rate were very high which exactly correlates with our results.

GA3 interferes with sugar signaling in endosperm and involves in plant regeneration by elongation of the embryos axis and accelerating the embryo maturation (31). The study of GA3 effect on the regeneration of *Brassica napus* L., showed that a higher concentration of GA3 could be effective on normal plantlets production and the medium without GA3 produced the lowest number of normal plantlets (32). Gibberellins by affecting auxin inhibit adventitious root formation. GA interacts with all other plant hormones, in some cases reciprocally, whereby GA affects but is also being affected by the other hormones. The direction and type (positive or negative) of the interaction depends on the biological process, tissue, developmental stage, and/or environmental conditions (33). In this study, we demonstrated that GA3 in the Golden Promise cultivar

was much higher than local cultivars (**Fig. 4B**). This finding may suggest that the amount of GA3 hormone and the percentage of regeneration are directly related. Different antioxidant systems have been shown to form in all cells to overcome the production and accumulation of ROS. ROS through interaction with hormone precursors plays an important role in their biosynthesis (34). ROS enhances root node production and auxin synthesis (35). Auxin plays an important role in elongation and cell division by producing ROS where it causes cell wall loosening and contributes to cell elongation and division (36). Hydrogen peroxide (H_2O_2) is a ROS contributing to root regeneration (37). By increasing the level of ROS, gibberellic acid synthesis is reduced (38). ABA is one of the factors that increase extracellular ROS production (39). In a recent report, CHIP-seq and RNA-seq analyses revealed that regulating genes of lateral root were involved in ROS metabolism and cell wall hydrolysis (40). In the present study, Iranian barley cultivars had much lower antioxidant levels and, of course, higher ROS levels than Golden Promise cultivar (**Fig. 6**). This may be the main reason for higher levels of ABA and IAA in barley cultivars that reduce regeneration and increase the formation of lateral roots.

Total phenols content of explants are one of the factors affecting tissue culture and regeneration efficiency (41). In a study on the effects of phenol content on the regeneration of sugarcane, Lorenzo (2001) demonstrated a close relationship between phenolic production and *in vitro* sugarcane shoot formation (42). Furthermore, in tissue culture of *Pinus sylvestris*, the regeneration rate was enhanced by increasing the production of shikimic acid (43). Our results showed that the total phenol content in Golden Promise was about 3 times higher than in the rest of the examined cultivars (**Fig. 7**). Generally, callus with higher concentrations of phenolic compounds showed a smaller size and compact consistency (**Fig. 1A**). According to Coenen and Lomax (1997), the accumulation of phenolic compounds correlates negatively with cell growth, which was also found in the current study (44). The amount of total phenol content seems to be directly related to the percentage of regeneration.

It is clear that endogenous hormones, antioxidants, and total phenols affect shoot regeneration in plant tissue culture. In this study, we demonstrated that these factors are variable in different cultivars of one species. In addition, we showed the optimal conditions of these factors in a standard cultivar, which had a high regeneration rate. Therefore, for successful regeneration of recalcitrant cultivars, external treatments should be

done in a way to reduce the inhibitory effects of internal factors. At least in case of barley, we need further studies to understand the effects of changes of external factors (plant growth regulators, antioxidants, etc.) on making a balance between endogenous factors in favor of shoot regeneration from callus tissue.

Acknowledgments

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