Enhancement of *Agrobacterium*-mediated transformation efficiency in immature embryo of *Triticum aestivum*, cv. Arya

Roghayeh Ahmadpour[1*], Nasser Zare[1], Rasool Asghari-Zakaria[1], Parisa Sheikhzadeh[1]

[1]Department of Agronomy and Plant Breeding, Faculty of Agriculture, University of Mohaghegh Ardabili, Ardabil, Iran.
**Corresponding author, E-mail: r.ahmadpour65@gmail.com. Tel: 0098-914-4544612.*

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Abstract
An efficient *Agrobacterium*-mediate transformation method was developed by employing different duration of sonication, *Agrobacterium* strains, type of inoculation medium and concentrations of acetosyringone in Arya cultivar of wheat. Immature embryos were used as an explant for inoculation with *Agrobacterium tumefaciens* harboring the recombinant pBI121 plasmid. Among the durations of sonication the highest percentage of GUS positive immature embryos (54.58 ± 1.14%) and transformation (0.78 ± 0.07%) was observed in 10-seconds of sonication. Among the *Agrobacterium* strains, the highest GUS expression was 62.50 ± 1.55% and 0.39 ± 0.04% with LBA4404 strain. Between types of inoculation medium, the highest GUS positive immature embryos and transformation (1.86 ± 0.14% and 0.75 ± 0.04%, respectively) was observed in using the IM inoculation medium. Between concentrations of acetosyringone, the highest transformation was 0.75 ± 0.04% obtained at 200 µM acetosyringone. Also, the studying of simultaneous effects showed that the highest transformation efficiency (1.56 ± 0.06%) obtained from immature embryos inoculated with LBA4404 strain followed by 10-seconds sonication, immature embryos inoculated with *Agrobacterium* in IM inoculation medium after 10-seconds sonication and immature embryos inoculated with *Agrobacterium* in inoculation medium containing 200 µM acetosyringone after 10-seconds of sonication.

Key words: Acetosyringone, *Agrobacterium*, Inoculation medium, Sonication, Transformation.

INTRODUCTION

Wheat is an important cereal and has a key role in economic development, food security and human nutrition (Li *et al*., 2012). This crop is cultivated on approximately 17% of the cultivatable lands and is an important source of calories and proteins for human (Jones, 2005). For functional genomic studies in plants, efficient genetic transformation system is an important strategy (Bakshi *et al*., 2011). *Agrobacterium*-mediated and micro-particle bombardment are efficient methods for transformation of plants (Supartana *et al*., 2006). *Agrobacterium*-mediated transformation has been used in many crops such as grain legumes. This method has several advantages than other methods. These advantages include the defined integration of transgenes, low copy number, and integration of foreign gene into transcriptional active regions of the plant chromosome (Hiei *et al*., 1994). However, using *Agrobacterium* for transformation has a few disadvantages. One of the most important disadvantage of this method is the organism's host specificity, resulting in low levels of transformation in certain plant species (Beranová *et al*., 2008). Cheng *et al*. (1997) reported the *Agrobacterium*-mediated transformation of wheat for the first time. Then, several studies were performed for transformation of wheat by *Agrobacterium* but the transformation efficiency was low (Jones, 2005). The studies showed that a limited number of wheat varieties have been transformed by *Agrobacterium*. This limitation is due to the differences in the abilities of callus induction and regeneration of

wheat varieties. Nevertheless, the *Agrobacterium*-mediated transformation of wheat is genotype-dependent (Supartana *et al*., 2006).

Several factors affect *Agrobacterium*-mediated transformation efficiency. The first is the strain of bacteria. According to the studies, in *Agrobacterium*-mediated transformations, DNA integration patterns are strain-dependent. The second factor is the type of tissue. Monocots and certain dicot tissues are not very receptive to *Agrobacterium*-mediated transformation. The third factor is application of acetosyringone. Acetosyringone is an inducer of T-DNA transfer and enhances the transformation efficiency. Therefore, with the manipulation of these factors it can be enhanced the transformation efficiency of plants (Trick and Finer, 1997).

Ultrasound has increased gene uptake by plant protoplast, cell suspension and intact tissues. Gene transfer by ultra-sonication is a good strategy and does not depend to the nature of theplant material (Liu *et al*., 2006). This method is called sonication assisted *Agrobacterium*-mediated transformation (SAAT) and enhance the efficiency of *Agrobacterium*-mediated transformation of recalcitrant plants (Bakshi *et al*., 2011). Exposure of the explants to short periods of sonication in the presence of *Agrobacterium* is to produce small and uniform micro wounds and channels across the tissue cells to permit *Agrobacterium* to penetrate more quickly into the membrane (Dutta *et al*., 2012). Trick and Finer (1997) reported that sonication assisted *Agrobacterium*-mediated transformation is an efficient *Agrobacterium*-based transformation technology for soybean and enhanced the transient expression of β-glucuronidase (*gus*). SAAT method has been successfully used in lobally pine, black locust, flax, citrus and banana (Tang *et al.*, 2001; Zaragozá *et al.*, 2004; Beranová *et al.*, 2008; Oliveira *et al.*, 2008; Subramanyam *et al.*, 2011). Therefore, the aim of this study was to investigate the effects of sonication, bacterial strain, inoculation medium and acetosyringone on *Agrobacterium*-mediated transformation of Arya cultivar of wheat.

MATERIALS AND METHODS

Plant materials and explants preparation

The seeds of Arya cultivar of wheat (*Triticum aestivum*) were obtained from the Seed and Plant Improvement Institute, Karaj, Iran. For immature embryo explant preparation, the seeds were planted in plots and maintained in a greenhouse at 21 ± 2ºC with 16/8 h light/dark photoperiod. The immature seeds were collected 20-25 days after pollination and surface sterilized with 70% (v/v) ethanol for 30-45 seconds, 2% (w/v) sodium hypochlorite solution for 13-15 min and rinsed three times with sterile distilled water.Then, the immature embryos were excised from sterilized immature seeds and cultured on MS induction medium (IM) supplemented with 2 mg/L 2,4-D and 200 mg/L caseine hydrolysate for 3 days for pre-induction.

Plasmid vectors and *Agrobacterium tumefaciens* strains

Two *A. tumefaciens* strains, EHA101and LBA4404 were used in this investigation. They carried the plasmid pBI121 containing the *β*-glucuronidase gene (*gus*) under the control of CaMV 35S promoter and *NOS* terminator and kanamycin resistant (*aadA*) gene for transformed bacteria and plant selection. Both strains were maintained on solid LB medium supplemented with 50 mg/L kanamycin and 50 mg/L rifampicin for transformed bacteria selection.

Agrobacterium-mediated transformation

A single colony from each strain was inoculated into 10 mL liquid LB medium with 50 mg/L kanamycin and 50 mg/L rifampicin antibiotics and grown over night at 28 °C with shaking (120 rpm). For investigating the effect of inoculation medium on transformation efficiency, when the final OD600 nm of the culture reached 0.9-1.2, bacterial cells were collected by centrifugation at 5000 rpm for 10 min, and re-suspended in liquid MS medium supplemented with 2 mg/L 2,4-D and 200 mg/L caseine hydrolysate (IM) and used for inoculation. Also, for investigation of the effect of acetosyringone (3′,5′-Dimethoxy-4′-hydroxyacetophenone, Sigma-Aldrich) on transformation efficiency, the bacterial cells were collected by centrifuge and re-suspended in the liquid MS medium supplemented with 2 mg/L 2,4-D, 200 mg/L caseine hydrolysate and 200 µM acetosyringone and used for inoculation.

SAAT treatment

To determine the optimum sonication time, immature embryo explants were immersed in 50 mL screw capped tubes containing 5 mL inoculation media (LA and IM) and placed at the center of a bath sonicator (Bandelin DT 255H, Germany). The explants were sonicated at a frequency of 37 kHz for 0, 10, 30 and 50 seconds and inoculated with *Agrobacterium* strains for 40 min. Then, the explants were co-cultivated with *Agrobacterium* on MS medium supplemented with 2 mg/L 2,4-D, 200 mg/L caseine hydrolysate for 3 days at 25 ± 1ºC in the dark.

Selection and regeneration

After 3 days of co-cultivation, the explantswere transferred into the selective callus induction medium

(MS medium supplemented with 2 mg/L 2,4-D, 200 mg/L caseine hydrolysate) containing 50 mg/L kanamycin and 400 mg/L cefotaxime at 25 ± 1ºC for 3 weeks in the dark. After 3 weeks, the produced embryogenic calli were transferred on the MS medium supplemented with 0.05 mg/L NAA, 25 mg/L kanamycin and 400 mg/L cefotaxime at 25 ± 1ºC with 16/8 h light/dark photoperiod for 2 weeks and the percentage of embryogenesis was measured. After 2 weeks, elongated and surviving shoots were transferred into the MS medium supplemented with 0.05 mg/L NAA, 400 mg/L cefotaxime without kanamycin and maintained at 25 ± 1ºC with a 16/8 h light/dark photoperiod for more growth and percentage of rooting and transformation was measured.

GUS histochemical assay

The GUS expression was assayed based on Altpeter *et al*. (2010). The percentage of GUS positive and GUS expression intensity was analyzed at the immature embryos after 3 days of co-cultivation. The GUS expression was analyzed in kanamycin resistant transgenic plant leaves. The explants were incubated in the GUS assay solution (solution 1: add 70 mg X-gluc (Sigma-Aldrich) to 2 mL of dimethyl sulfoxide and solution 2: 150 mL of 100 mM Na_3PO_4 with 5 mL of 0.5 M EDTA and 200 µL of Triton X-100. Solutions 1 and 2 were mixed and and the final volume was made to 200 mL with ddH_2O) and samples were kept for 16 h at 37ºC in the dark and then were observed under stereo microscope and GUS expression and intensity were measured. To record GUS expression intensity, zero was considered as no expression, 0.01-1 as low expression, 1.01-2 as relatively low expression, 2.01-3 as medium expression, 3.01-4 as relatively high expression and 4.01-5 as high expression.

Statistical analysis

The percentage of embyogenesis, rooting, GUS positive, GUS expression intensity and percentage of transformation were performed in four replicates and analyzed by SPSS 22.0 statistical software.

RESULTS AND DISCUSSION

Effect of sonication

The results showed that sonication duration had an effect on the percentage of embryogenesis, percentage of rooting, percentage of GUS positive, GUS expression intensity and percentage of transformation (Table 1). In this study, the application of sonication has a negative effect on embryogenesis after inoculation of immature embryos with *Agrobacterium*. With increasing of sonication duration, percentage of embryogenesis decreased. Therefore, control treatment had the highest percentage of embryogenesis. Sonication increased percentage of rooting compared to the control but increasing sonication duration caused the percentage of rooting to decrease. The highest percentage of rooting was 30.27 ± 1.72% at 10-seconds of sonication. The inoculated immature embryos with *Agrobacterium* were assayed histochemically for GUS expression after 3 days of co-cultivation (Figure 1). Sonication for 10-seconds produced 54.58 ± 1.14% GUS positive immature embryos and beyond 10-seconds, sonication decreased the percentage of GUS positive immature embryos, however, they were higher than the control. The GUS expression intensity increased by sonication treatment. The 50-seconds sonication presented a moderate expression of GUS that was higher than other treatments, as the10 and 30-seconds of sonication and control had low expression of GUS. The highest effect of sonication on transformatio-n efficiency was observed at 10-seconds. However, 30 and 50-seconds of sonication also demonstrated a positive effect on transformation. Among various sonication durations, 10-seconds produced the highest plant transformation (0.78 ± 0.07%) (Table 1). Efficient *Agrobacterium*-mediate transformation was affected by several factors such as efficient interaction between *Agrobacterium* and host tissue. In this study, sonication enhanced interaction between *Agrobacterium* and immature embryos. Soication of tissue during infection with *Agrobacterium* increases transformation efficiency by producing small and uniform wounds, in which wounds cause the secretion of more phenolic compounds from tissue, activate *vir* genes interactions and facilitates T-DNA transfer (Santarem *et al*., 1998; Beranová *et al*., 2008). Longer duration of sonication have inhibitory effect on plant cells, such as immediate cell lysis, suppression of RNA and protein synthesis of cell walls (Joersbo and Brunstedt, 1992). Therefore, short duration with a low energy of ultrasound causes of transformation in SAAT treatment to increase (Santarem *et al*., 1998). SAAT has been shown to provide efficient delivery of T-DNA into plant cells in *Leptadenia pyrotechnica* (Dutta *et al*., 2012), *Linumus itatissimum* L. (Beranová *et al*., 2008), cowpea (Bakshi *et al*., 2011) and chickpeas (Pathak and Hamzah, 2008).

Effect of *Agrobacterium* strain

Results indicated that the type of *Agrobacterium* strain has an important role in the percentage of embryogenesis, rooting, GUS positive immature embryos, GUS expression intensity and efficiency of wheat transformation (Table 2). Two *Agrobacterium* strains (LBA4404 and EHA101) were used in this

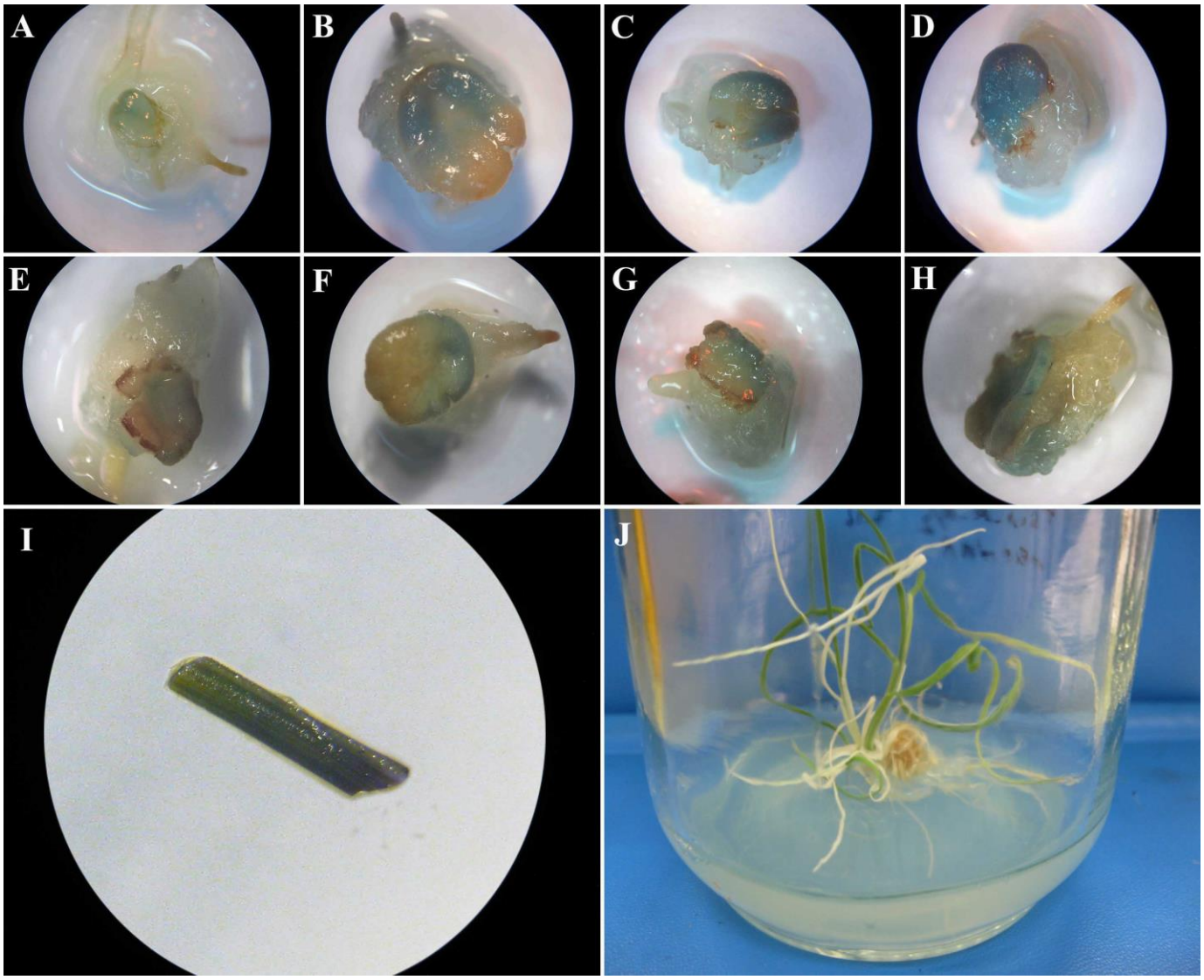

Figure 1. Transformation and regeneration of plantlets from immature embryos of Arya cultivar. **A-D:** Transient GUS expression in immature embryos inoculated by LBA4404 strain at 0, 10, 30 and 50 seconds of sonication, respectively. **E-H:** Transient GUS expression in immature embryos inoculated by EHA101 strain at 0, 10, 30 and 50 seconds of sonication, respectively. **I:** GUS expression in transformed plantlet leaf after selection. **J:** Transformed regenerated plantlet.

Table 1. Effect of different sonication durations on the percentage of embryogenesis, rooting, GUS positive, GUS expression intensity and percentage of transformation of Arya cultivar of wheat.

Sonication (s)	Percentage of embryogenesis	Percentage of rooting	Percentage of GUS positive	GUS expression intensity	Percentage of transformation
0	91.74±1.18	19.46±1.28	31.25±1.44	1.57±0.16	0.00±0.00
10	85.66±1.58	30.27±1.72	54.58±1.14	1.78±0.24	0.78±0.07
30	83.27±1.82	25.91±1.05	50.83±1.63	1.66±0.15	0.42±0.04
50	78.67±1.79	21.85±1.96	47.50±1.56	2.07±0.17	0.31±0.03

Table 2. Effect of *Agrobacterium* strains on the percentage of embryogenesis, rooting, GUS positive, GUS expression intensity and the percentage of transformation of Arya cultivar of wheat.

Strains	Percentage of embryogenesis	Percentage of rooting	Percentage of GUS positive	GUS expression intensity	Percentage of transformation
LBA4404	97.09±0.77	33.61±1.84	62.50±1.55	2.04±0.11	0.39±0.04
EHA101	72.58±1.18	15.13±1.14	29.58±1.57	1.50±0.14	0.36±0.02

Table 3. The simultaneous effect of sonication and *Agrobacterium* strain on the percentage of embryogenesis, rooting, GUS positive, GUS expression intensity and the percentage of transformation of Arya cultivar of wheat.

Agrobacteruim Strain	Sonication (s)	Percentage of embryogenesis	Percentage of rooting	Percentage of GUS positive	GUS expression intensity	Percentage of transformation
LBA4404	0	96.04±1.93	30.67±1.57	41.67±1.33	1.97±0.02	0.00±0.00
	10	95.96±1.74	42.06±1.97	83.33±1.27	2.39±0.03	1.56±0.06
	30	98.53±0.96	28.19±1.61	75.00±0.63	2.00±0.02	0.00±0.00
	50	97.83±1.49	33.50±1.27	50.00±1.95	1.81±0.02	0.00±0.00
EHA101	0	87.45±2.33	8.24±1.42	20.83±1.16	1.17±0.02	0.00±0.00
	10	75.78±2.79	18.47±1.52	25.83±1.01	1.17±0.03	0.00±0.00
	30	68.02±2.58	23.62±1.11	26.67±1.77	1.33±0.02	0.83±0.04
	50	59.51±2.07	10.20±0.98	45.00±1.24	2.33±0.03	0.62±0.03

study. Between them, the LBA4404 was found to be more effective. LBA4404 produced the highest percentage of embryogenesis (97.09 ± 0.77%). Also, this strain produced the highest rate of rooting than EHA101. The 33.61 ± 1.84% of inoculated immature embryos with LBA4404 strain produced root, whereas this rate for EHA101 was 15.13 ± 0.14%. Therefore, the percentage of rooting in LBA4404 was 2-fold higher than EHA101. The LBA4404 caused the production of the highest GUS positive immature embryos at a rate of 62.50 ± 1.55% efficiency, whereas EHA101 produced 29.58 ± 1.57% of GUS positive immature embryos. In other words, the LBA4404 produced 2-fold more GUS positive immature embryos over EHA101. The analysis of GUS expression intensity showed that the transient GUS expression in immature embryos inoculated with LBA4404 was higher than EHA101. The transient GUS expression in immature embryos inoculated with LBA4404 was moderate, whereas in immature embryos inoculated with EHA101 was relatively low (Figure 1). In this study, the transformation efficiency was affected by *Agrobacterium* strains. The transformation efficiency in LBA4404 was higher than EHA101, as the rate of transformation in LBA4404 and EHA101 was 0.39 ± 0.04% and 0.36 ± 0.02%, respectively (Table 2). The important internal factors that influence the infecting ability of *A. tumefaciens* are chromosome and activating potency of genes in virulence region (Subramanyam *et al*., 2011). It has been reported that, LBA4404 and EHA101 have different chromosomal background and *vir*-helper plasmid with different levels of activating potency (Hood *et al*., 1993). It was likely for these reasons that LBA4404 had a stronger ability to infect wheat Arya cultivar than EHA101. Akama *et al*. (1992) proved that EHA101strain had the highest efficiency of regeneration of transformed shoots in *Arabidopsis thaliana*. Lulsdorf *et al*. (1991) showed that LBA4404 and EHA101 were suitable for pea transformation. Tsukazaki *et al*. (2002) reported that LBA4404 produced a higher number of GUS positive explants of cabbage than EHA101.

Simultaneous effects of sonication and *Agrobacterium* strain

In this study, the simultaneous effect of sonication and *Agrobacterium* strain was investigated for the first time. The results showed that simultaneous application of sonication and different *Agrobacterium* strains affected the percentage of embryogenesis, rooting, GUS positive, GUS expression intensity and percentage of transformation (Table 3). The immature embryos inoculated with LBA4404 strain followed by 30-seconds of sonication had the highest percentage of

Table 4. Effect of inoculation medium on the percentage of embryogenesis, rooting, GUS positive, GUS expression intensity and the percentage of transformation of Arya cultivar of wheat.

Strains	Percentage of embryogenesis	Percentage of rooting	Percentage of GUS positive	GUS expression intensity	Percentage of transformation
IM*	85.82±2.72	28.53±1.03	50.00±2.46	1.86±0.14	0.75±0.04
LB	83.85±3.60	20.21±1.63	42.08±2.44	1.68±0.13	0.00±0.00

* MS medium supplemented with 2 mg/L 2,4-D and 200 mg/L caseine hydrolysate.

Table 5. The simultaneous effect of sonication and inoculation medium on the percentage of embryogenesis, rooting, GUS positive, GUS expression intensity and the percentage of transformation of Arya cultivar of wheat.

Inoculation medium	Sonication (s)	Percentage of embryogenesis	Percentage of rooting	Percentage of GUS positive	GUS expression intensity	Percentage of transformation
IM	0	84.88±2.69	17.22±2.13	33.33±1.54	1.53±0.03	0.00 ±0.00
	10	91.65±1.92	30.90±1.03	58.33±1.36	1.97±0.03	1.56±0.06
	30	86.95±2.01	39.03±1.11	54.17±1.03	1.89±0.02	0.83±0.04
	50	79.80±3.90	26.98±1.83	54.17±1.90	2.06±0.03	0.63±0.03
LB	0	98.61±1.39	21.69±1.52	29.17±1.16	1.61±0.02	0.00±0.00
	10	79.67±3.27	29.63±1.71	50.83±1.21	1.58±0.04	0.00±0.00
	30	79.60±3.27	12.79±1.10	47.50±1.29	1.44±0.02	0.00±0.00
	50	77.54±3.03	16.72±1.65	40.83±1.74	2.08±0.01	0.00±0.00

embryogenesis (98.53 ± 0.96%). The maximum percentage of rooting was observed in immature embryos inoculated with LBA4404 fallowed by 10-seconds of sonication. The highest GUS positive immature embryos were obtained after inoculation with LBA4404 strain followed by 10-seconds of sonication. The highest GUS expression intensity was observed in immature embryos inoculated with LBA4404 strain after 10-seconds of sonication. Also, the highest transformation efficiency was 1.56 ± 0.06% after inoculation with LBA4404 strain followed by 10-seconds of sonication. Therefore, the inoculation with LBA4404 after 10-seconds of sonication had a higher effect on transformation in Arya cultivar of wheat (Table 3).

Effect of inoculation medium

The type of inoculation medium had a more effect on the percentage of embryogenesis, rooting, GUS positive and transformation efficiency (Table 4). In this study two types of media including LB and IM were used for inoculation. The results showed that, the IM inoculation medium was more effective than LB. The percentage of embryogenesis and rooting in IM inoculation medium was 85.82 ± 2.72% and 28.53 ± 1.03%, respectively. Also, IM inoculation medium produced 50 ± 2.46% GUS positive immature embryos, but this rate with LB medium was 42.08 ± 2.44%. The analysis of transient GUS expression indicated that, both inoculation media had about the same ratio of gene expression intensities. The highest rate of transformation was obtained in the IM inoculation medium (0.72 ± 0.04), whereas the rate of LB inoculation medium was 0%. Therefore, IM inoculation medium is better than LB for Arya cultivar transformation (Table 4). According to other studies, the composition of the inoculation medium had a significant effect on the transformation efficiency of tomato (Davis *et al.*, 1991; Wu *et al.*, 2006; Rai *et al.*, 2012) and citrus (Pena *et al*., 2004).

Simultaneous effects of sonication and inoculation medium

Here the simultaneous effect of sonication and inoculation medium is reported for the first time. The non-sonicated immature embryos inoculated with *Agrobacterium* in LB inoculation medium demonstrated 98.61 ± 1.39% of embryogenesis (Table 5). The highest percentage of rooting was 39.03 ± 1.11% which was obtained in immature embryos sonicated for 30-seconds and inoculated in the IM inoculation medium. The immature embryos inoculated with *Agrobacterium* in the IM inoculation medium followed by 10-seconds of

Table 6. Effect of acetosyringone on the percentage of embryogenesis, rooting and transformation of Arya cultivar of wheat.

Acetosyringone (µM)	Percentage of embryogenesis	Percentage of rooting	Percentage of transformation
0	83.85±2.42	28.53±2.03	0.40±0.02
200	88.48±2.69	47.96±2.72	0.75±0.04

Table 7. The simultaneous effect of sonication and acetosyringone on the percentage of embryogenesis, rooting, GUS positive, GUS expression intensity and the percentage of transformation of Arya cultivar of wheat.

Acetosyringone (µM)	Sonication(s)	Percentage of embryogenesis	Percentage of rooting	Percentage of transformation
0	0	79.67±2.58	17.22±1.03	0.00±0.00
	10	79.59±2.40	30.90±2.23	1.02±0.02
	30	77.54±2.71	39.03±2.10	0.59±0.01
	50	98.61±0.93	26.98±2.52	0.00±0.00
200	0	76.90±2.36	24.81±2.51	0.00±0.00
	10	91.22±1.69	59.86±2.65	1.56±0.04
	30	96.55±1.15	56.91±2.89	0.83±0.02
	50	89.26±2.55	50.27±2.89	0.58±0.02

sonication had the highest percentage of GUS positive (58.33 ± 1.36%). The highest GUS expression intensity was moderate, which was obtained in the immature embryos inoculated with *Agrobacterium* in LB inoculation medium after 50-seconds of sonication. The maximum transformation efficiency was 1.56 ± 0.06% which was observed in 10-seconds of sonicated immature embryos inoculated in the IM inoculation medium (Table 5).

Effect of acetosyringone

In the present study, we used 200 µM acetosyringone into inoculation medium (IM) for increasing transformation efficiency. The results indicated that, the addition of acetosyringone on inoculation medium affected embryogenesis, rooting and transformation efficiency. Application of acetosyringone increased percentage of embryogenesis from 83.85 ± 2.42% in control to 88.48 ± 2.69%. In other word, using acetosyringone in the inoculation medium exposed a positive effect on embryogenesis. Also, using acetosyringone increased the percentage of rooting about 2-fold over control (i.e 47.96 ± 2.72% VS 28.53 ± 2.02%) (Table 6). Addition of acetosyringone had a positive effect on transformation efficiency, in which the ratio was raised from 0.40 ± 0.02% (in control) to 0.75 ± 0.04% (Table 6). Therefore, the acetosyringone plays an important role in transformation of wheat. *Agrobacterium* attacks wounded plants in response to phenolic compounds such as acetosyringone and α-hydroxy acetosyringone are released by the plant cells. These compounds activate the *vir* genes present on the Ti plasmid of *A. tumefaciens*. But, monocotyledon plants such as wheat are not producing these compounds. Hence, the exogenous application of acetosyringone in the inoculation and co-cultivation media improve the transformation efficiency (Subramanyam *et al*., 2011). Hiei *et al*. (1994) demonstrated that acetosyringone at 100 µM of concentration had an important effect transformation of rice. Tripathi *et al.* (2010) reported that by using 350 µM acetosyringone they achieved a high transformation frequency in rice.

Simultaneous effects of sonication and acetosyringone

The simultaneous effect of sonication and acetosyringone has not been reported before. The 50-seconds sonicated immature embryos inoculated with *Agrobacterium* in inoculation medium without acetosyringone had 98.61±0.93% embryogenesis. The maximum percentage of rooting was about 59.86±2.65% observed at immature embryos sonicated for 10-seconds and inoculated with *Agrobacterium* in inoculation medium (IM) containing 200 µM acetosyringone. The highest transformation efficiency

was 1.56 ± 0.04% obtained in immature embryos inoculated with *Agrobacterium* in the inoculation medium containing 200 µM acetosyringone followed by 10-seconds of sonication (Table 7). Chugh *et al.* (2012) inoculated bread and pasta wheat with *Agrobacterium* in the presence of 200 µM actosyringone and reported transformation efficiencies of 1.16% and 0.84%, respectively. Patnaik *et al.* (2006) used 200 µM acetosyringone in bacterial growth medium, inoculation and co-culativation medium for increasing transformation effeciency in wheat and reported that the transformation efficiency ranged from 1.28 to 1.77%. Therefore, increasing the transformation effeciency in this study was more pronounced than those of other studies in the presence of acetosyringone. This observation may be due to the affect of sonication.

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