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IMPROVING *AGROBACTERIUM* MEDIATED TRANSFORMATION PROTOCOL FOR INTEGRATION OF *XA21* GENE IN WHEAT (*TRITICUM AESTIVUM* L.)

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Abstract

During the present study, embryo derived calli of 3 wheat varieties viz., Chakwal-97, Inqulab-91 and Tartara-2000 were infected with *Agrobacterium tumefaciens* strain EHA101 carrying genes *Xa21* for bacterial blight resistance. Hygromycin @ 50mg/l was lethal dose for the selection of transformed calli. Up to 90% GUS expression was observed in selected calli on the selection media containing 50 mg/l hygromycin. The highest number (90%) of GUS expression was observed from 21 days old calli. Callus differentiation was not observed at 0-50µM application, hygromycin resistant calli were obtained from the calli treated with acetosyringone (200-400µM). The application of 400 µM acetosyringone promoted the production of resistant calli and GUS expression was found to be higher in Inqulab-91 (90%). A combined treatment of cefotaxime (500mg/l) and carbenicillin (250mg/l) are better than cefotaxime (500mg/l) or carbenicillin (500mg/l) alone for the control of bacteria. Transient GUS expression was observed in calli of all varieties. Stable GUS expression was observed in transformed leaves and roots of all varieties especially Tartara-2000 and Inqulab-91. The transformation was also confirmed by the presence of 1.4kbp band in PCR and Real time quantitative PCR in two successive generations of wheat.

Introduction

Many biotic factors are responsible for low yield of wheat. Diseases caused by bacteria and fungi are the major biotic factors to decrease wheat yield. Conventional plant breeding usually involves the production of variability by making sexual crosses between selected genotypes possessing characters to be combined, to produce a population of plants that include superior genotypes. Availability of diversified germplasm is the basic requirement for the success of any breeding program. (Akram *et al.*, 1997). By utilizing transformation techniques, wheat varieties can be improved to resist against heat, drought, diseases and salt stress etc.

In *Agrobacterium*-mediated transformation of cereals, a number of factors such as genotype, explant, *Agrobacterium* strain, binary vector, selectable marker gene and promoter, inoculation and co-culture conditions, inoculation and co-culture medium, osmotic treatment, desiccation, *Agrobacterium* density and surfactants, tissue culture and regeneration medium may influence the recovery of stable plant cells after *Agrobacterium* infection (Shrawat & Lörz, 2006, Cheng *et al*., 2004). The aim of the present study was to improve of transformation protocol in three wheat varieties viz., Chakwal-97, Inqulab-91 and Tartara-2000 by utilizing *Agrobacterium tumefaciens* strain EHA101 with broad spectrum bacterial disease resistant gene *Xa21*.

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Materials and Methods

Explant source: Three weeks old embryogenic and compact calli (3-5 mm in diameter) of 6 wheat varieties were co-cultivated with *Agrobacterium* in each experiment for transformation.

***Agrobacterium* strain and plasmid:** Transformation of wheat calli was carried out by using *Agrobacterium tumefaciens* strain EHA101 (Hood *et al*., 1986) harbouring pTCL5 plasmid (Baisakh *et al*., 2000). pTCL5 a binary vector containing *Xa 21* gene (disease resistant), hygromycin resistance gene and an intron- ß -Glucuronidase Coding Region (GUS)-gene in T-DNA region. The gene for GUS has intron in the middle of the coding region and is driven by 35S promoter of cauliflower mosaic virus (Ohta *et al*., 1990). This intron-GUS reporter gene expresses GUS in plant cells but not in the cells of *A. tumefaciens*.

Culture of *Agrobacterium*: The *Agrobacterium* strain EHA101 containing the binary vector pTCL5 was grown in YEP medium (An *et al*., 1998) supplemented with antibiotics such as Canamycin (50 mg/l) and Rifampicin (10mg/l) (Baisakh *et al*., 2000) in 250 ml Erlenmeyer flask. The culture was grown at 105-110 rpm on a rotary shaker at 28^{o}C for 16 h. Aliquots of *Agrobacterium* culture were pipetted out and the optical density of the culture was measured at 600nm (OD_{600} of 1-1.5) (Khan *et al*., 2003). The 18-hour grown culture was used for all the experiments. Overnight culture of *A. tumefaciens* having an OD_{600} of 1-1.5 was centrifuged at 3000 rpm for 15 min. Supernatant was discarded and pellet was resuspended in Callus Induction Medium (CIM)1+Acetosyringone media $_{(liq)}$ (50- 400µM) and mixed on vortex.

Determination of lethal dose of hygromycin: To check the lethal dose of hygromycin, three weeks old scutellum derived calli of all wheat varieties were subjected to hygromycin selection media containing MS salts and vitamins supplemented with 3 mg/l 2,4-D, 30g/l sucrose and 8g/l agar. Different doses of hygromycin @ 25, 50 and 75 mg/l were added. The cultures were incubated for four weeks at 25˚C ± 2˚C with 10-h photoperiod at light intensity of 48 µ mol m^{-2} s^{-1} supplemented with cool white fluorescent tubes. The results were recorded after two weeks.

Co-cultivation: To improve the transformation efficiency of six wheat varieties, increasing concentration of acetosyringone were added in co-cultivation medium to achieve final concentration of 50µM, 100µM, 200µM, 300 µM and 400µM supplemented with Amino Acid. Three week old scutellum derived embryogenic compact calli were immersed in bacterial suspension for 2-3 min. To remove excess of bacteria soaked calli were blotted dry with sterile blotting paper. Then calli were transferred to piece of filter paper placed on a co culture medium (CIM2 (solid) + Acetosyringone 400µM (Toriyama *et al.*, 1988).

Callus proliferation and evaluation: A growth index (G.I) was prepared with different concentrations of acetosyringone at 50µM, 100µM, 200µM, 300µM and 400µM according to Matsumoto & Fukui (1998). The degree of differentiation of the seed derived calli after 3-4 weeks of transformation to the selection medium is as under:

0; Callus did not differentiate.1; Callus differentiation occurred half of the original size2; Callus differentiation occurred double of the original size3; Callus differentiation occurred three times of the original size.

Selection: After co-cultivation the infected calli were washed with liquid callus induction medium + Carbinciline (Cb) and Cefataxmine (Cf). Hygromycin was used to select transformed calli from the non-transformed ones and Carbinciline (Cb) and Cefataxmine (Cf) were added to kill any *Agrobacterium* cells still attached to the surface of calli. These calli were blotted dry with sterile filter paper and then transferred to CIM2 –Cb and Cf media for pre-selection. After one week of pre-selection period, these calli were transferred to CIM2 - Hyg Cb and Cf medium for selecting transformants.

Direct selection was done after co-cultivation period. CIM1 $_{(liq)}$ - Hyg Cb and Cf was used as washing media and calli were transferred to CIM2 $_{(solid)}$ - Hyg + Cb and Cf. Selection was done for two weeks.

Regeneration: Two weeks after selection transformed calli were transferred to regeneration media that is RM –Hyg + Cb and Cf for shoot regeneration and root development. The regenerated shoots were further transferred to RM2 - Hyg + Cb and Cf for full plant development with extensive root system.
Transformation efficiencies were calculated in two ways;

Transformation efficiency based on callus number (TEC)

$$\text{TEC (\%)} = \frac{\text{Number of calli regenerated}}{\text{Number of calli independently inoculated}} \text{ X } 100$$

Transformation efficiency based on plant number (TEP)

$$\text{TEC (\%)} = \frac{\text{Number of plant obtained}}{\text{Number of calli independently inoculated}} \text{ X } 100$$

Assay of β-glucuronidase (gus) activity: The GUS assay was carried out essentially as described by Jefferson, (1987). Calli were incubated in X-Gluc solution containing 1mg/l 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.5% triton X-100, 20% methanol and 50mM sodium phosphate buffer (pH 7.0). To compare transient and stable T-DNA transformation, GUS analysis was done.

a. Transient GUS expression: For observing transient GUS expression, calli immediately after co-cultivation was dipped in GUS staining solution.

b. Stable GUS expression: For observing stable GUS expression, four weeks old transformed calli and regenerated plants were assayed by dipping transformed calli, transformed shoot, root and leaf into GUS staining solution. The reaction mixture was incubated at 37˚C for 2-3 days and calli were examined under microscope.

Results

Sensitivity to hygromycin: Prior to transformation, an effective concentration of antibiotic for the selection of transformed cells was determined by culturing calli on N6 medium containing various concentrations of hygromycin (0, 25, 50, 75 and 100mg/l). Calli became dry and yellow on N6 medium containing 50mg/l of hygromycin (Fig. 1). Hygromycin at concentration of 75, and 100 mg/l caused complete necrosis of the calli after three weeks. At 25 mg/l only 26% calli turned brown and 60 % calli exhibited growth. While at 50 mg/l, 79% calli turned brown and no callis growth was observed (Table 1). Therefore 50mg/l of hygromycin was considered effective dose for selection of transformants.

Age of calli: Statistically significant differences in average number of calli expressing GUS from different ages (15, 21, 28 and 35 days) co-cultivated for two days with *A. tumefaciens* was observed among genotypes and also among treatments. The variety x treatment interaction was also significant (Fig. 2). Keeping other conditions constant that is pH of co-culture medium at 5.8, one third of co-cultured calli were incubated with GUS substrate immediately after co-cultivation and the remaining were transferred to selection medium N_6 +2, 4 -D 2mg/l + 50mg/l hyg + 500mg/l cefotaxime and 250 mg/l carbenicillin (Table 2). The highest number of GUS expression was observed from 21 days old calli followed by 14 days old calli. GUS expression was also observed in 28 days (10 %) old calli, but no GUS expression was observed in 35 days old calli.

Time for co-cultivation: Table 3 showed the insignificantly different effect of period of co-culture on transient GUS expression of 21 days old wheat calli in six wheat varieties but the means of treatment among genotypes was significant. The average number of calli showed GUS expression (7.2), when co-cultivation time was kept for two days and normal bacterial growth was observed. GUS expression was decreased as the period was increased and excessive bacterial growth was observed. The lowest average number of calli showed GUS expression, when co-cultivation time period was kept one day and bacterial growth was below normal. GUS expression was not observed in cultures co-cultivated for four days. The tissues were adversely affected during prolonged co-cultivation period. It is clear from the result that to obtain efficient expression of GUS in wheat, 2 days co-cultivation with *Agrobacterium* was needed.

Effect of acetosyringone: To improve transformation efficiency acetosyringone in different concentrations viz. 50, 100, 200 and 400 µM were used in four experiments. In one experiment acetosyringone (50-400µM) was included in both the liquid medium (CIM1 + acetosyringone) (Table 3) as well as in solid bottom medium (CIM2 + acetosyringone) (Table 3). While in rest of the three experiments acetosyringone was omitted from either of the two media or from both. When the acetosyringone was omitted from either of the two media solely or from both of the liquid and solid bottom media, no GUS expression was observed and transformation efficiency became zero. GUS expression was 90% when both liquid and basal solid media, had acetosyringone, indicating that acetosyringone was found to be necessary for the transformation protocol in wheat (Table 4).

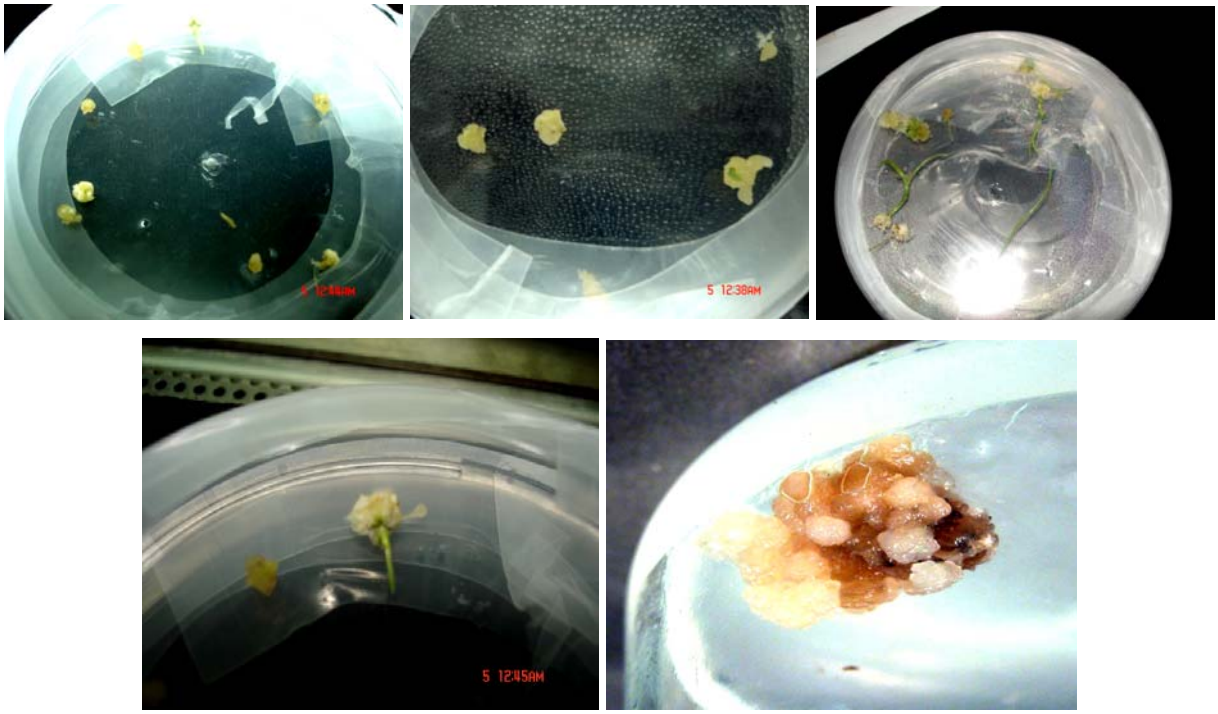

Fig. 1. Selection of calli on Hygromycin concentration
a. 0mg/l, b. 25mg/l, c. 50mg/l, d. 75mg/l, e. 100mg/l

Fig. 2. Effect of age of calli on GUS expression after two days of co-cultivation in wheat (*Triticum aestivum* L.)
a. 14 days old scutellum derived calli, b. 21 days old scutellum derived calli, c. 28 days old scutellum derived calli

After co-cultivation, calli were transferred to the selection medium containing callus induction medium with 50 mg/l hygromycin and 500 mg/l cefotaxime along with 250 mg/l carbencillin. After 2-3 weeks on selection medium calli showed sustained proliferation at one or more regions. Continuous selection on hygromycin containing medium resulted in the increase of proliferation apparently resistant embryogenic calli. Maximum number of calli showing blue color with maximum frequency was found when calli co-cultivated on co-cultivation medium 5 (N6 basal, 2,4-D 2mg/L, sucrose 30g/l gelrite 4g/l, pH 5.8, acetosyringone 400µM). It was evident that increase in concentration of acetosyringone enhances the GUS activity (Fig. 3 & 4).

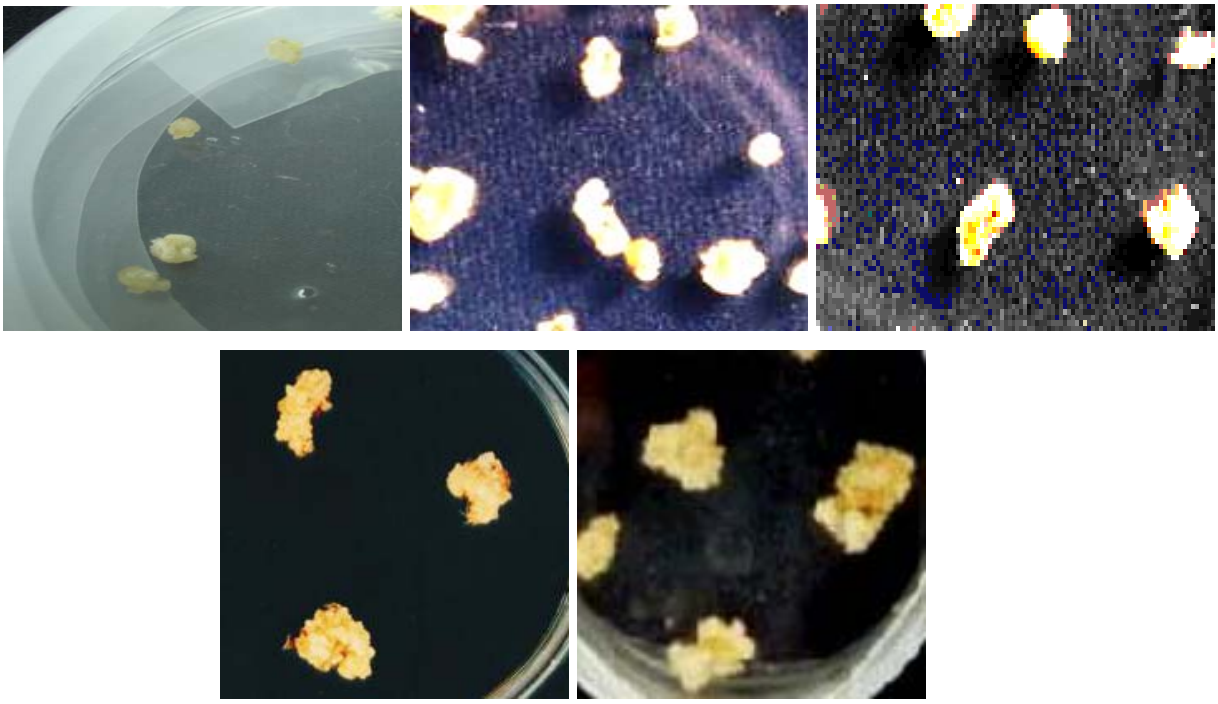

Fig. 3. Degree of differentiation of callus of wheat after 15-21 days on N_6 medium with Acetosyringone at concentration of a) 50 µM, b) 100µM, c) 200 µM, d) 300 µM, e) 400 µM

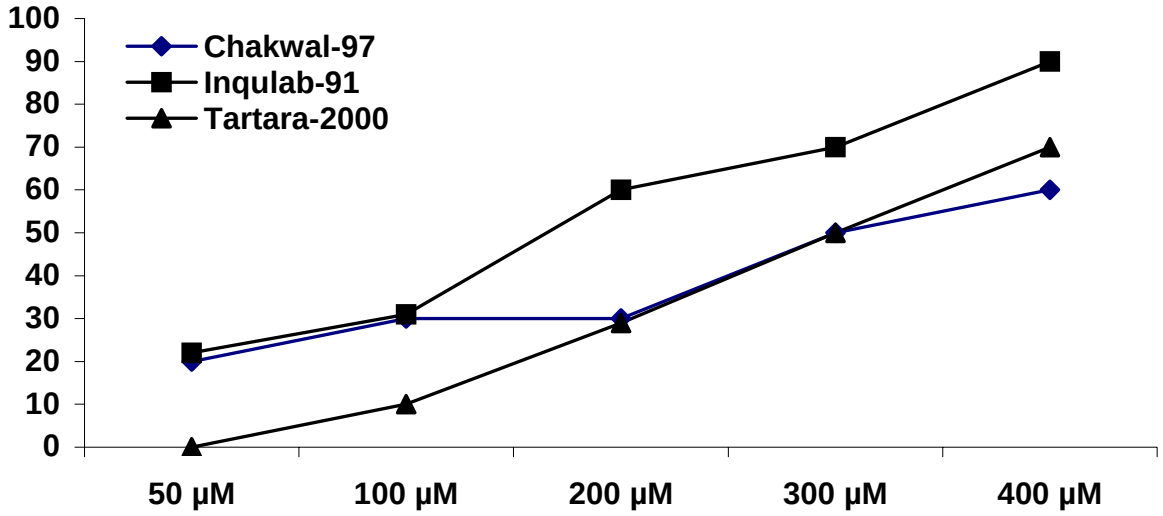

Fig. 4 Percent GUS expression among six different wheat varieties at different concentration of Acetosyringone.

At the application of 50µM acetosyringone, none of the calli of wheat varieties inoculated with *Agrobacterium* proliferated on the selective medium. The resistant calli only emerged by the application of higher concentrations of acetosyringone (Fig. 5). When 100 µM acetosyringone was used, Inqulab-91, Chakwal-97 and Tartara-2000 proliferated and differentiated to G.I.1 on the other hand only Inqulab-91reached up to G.I.2 (Table 3).

When 200 µM acetosyringone was applied, 50 out of 66 calli of Chakwal-97 and Tartara-2000 showed G.I.2, other remaining 16 calli of Inqulab-91 showed growth and proliferation up to G.I.1 (Table 4).

At the concentration of 300 µM acetosyringone variation was observed. Inqulab-91 showed growth and proliferation up to G.I.3. Acetosyringone at the concentration of 400 µM, increased proliferation and size of calli three times to the original size. 41 calli of Chakwal-97 and Tartara-2000 out of 125 reached up to G.I.3, while 84 calli of Inqulab-91 showed G.I.2 (Table 5). It was concluded that at higher concentration of acetosyringone 200-400 µM calli continued to grow, therefore it was considered that they consisted of transformed cells (Fig. 6 & Table 5).

Effect of cefotaxime and carbenicillin: Effect of antibiotics cefotaxime (500mg/l) or carbenicillin (500mg/l) or both in selection (after 2 days of co-cultivation) and regeneration medium (after 15 days of selection) of transformed wheat calli showed significant difference among genotype, treatment and interaction among variety x treatment. Significantly higher numbers of calli (10.88) were observed with cefotaxime 500 mg/l along with carbencillin 250mg/l compared to 500mg/l carbenicillin (average 7.77) and of 500mg/l cefotaxime (average 5.44) alone.

It means that both cefotaxime (500mg/l) and carbenicillin (250mg/l) are better than cefotaxime (500mg/l) or carbenicillin (500mg/l) alone for the control of bacteria (Table 6).

Comparison of two different protocols of selection: Calli derived from scutellum of different wheat varieties were co-cultivated for two days with *A. tumefaciens* strain EHA101 carrying *Hpt* gene, *Xa 21* gene and *β. glucoronidase* gene. Calli were then screened by following two selection protocols. In first protocol pre-selection was also done before selection. After co-cultivation calli were placed on the pre-selection media N_6 + 2,4-D + carbenicillin + cefotaxime for one week. Then hygromycin resistant calli were transferred to new selection media N_6 +2, 4-D + carbenicillin + cefotaxime + Hygromycin for two weeks (Table 7). In second protocol direct selection was done that is calli after two days of co-cultivation were transferred to selective media for two weeks after giving washing to calli, for 2-3 min., with N6 +2,4-D + carbenicillin + cefotaxime + Hygromycin. The results indicate that these two different protocols of selection are significantly different at 0.01 level of probability (Table 7). On the other hand the interaction between genotype and medium was not significantly different at 0.01%. Average number of resistant calli produced was significantly higher in direct selection as compared to indirect selection (Table 8).

GUS analysis: To compare transient and stable T-DNA transformation, GUS analysis was carried out immediately after co-cultivation as well as in calli 4 week after transformation. Transient GUS expression was observed in calli of all varieties.

For stable GUS expression, four weeks old transformed and un-transformed calli were assayed. Calli that developed from untransformed explant (seeds) showed no detectable GUS activity, while calli of transformed explant stained blue (Fig. 8).

Compared to the 90% of the explant showing GUS activity after two days of co-cultivation, only 40% explants developed hygromycin resistant calli and showed GUS^+ assay after four weeks. Each variety showed stable GUS expression leaves and roots of some of its transformed plants while in Inqulqb-91 all plants produced showed stable GUS expression. The segments of roots resulted in more deep blue staining in comparison to leaves (Figs. 5 & 6).

Regeneration frequency of transgenic plants and their GUS expression: The primary embryogenic callus induced from mature seed was used for *Agrobacterium*-mediated transformation of various wheat varieties (Fig. 6). After 2-days of co-cultivation, calli transferred to selection medium (N6 + Hygromycin + carbenicillin + cefotaxime) containing 50mg/l hygromycin grew slowly during the first selection period of two weeks. However from the third week, the calli that seemed to be Hygromycin resistance showed growth and proliferation on the selection medium.

After selection on selection medium (N6 + 2, 4-D at 2 mg/l + hygromycin + carbenicillin + cefotaxime), the hygromycin resistant (50mg/l) calli from all varieties were transferred to the regeneration medium. The regeneration medium for each variety was selected on the basis of experiment done to optimize regeneration medium for control plants (Fig. 6).

After two-three week, the transformed plants were readily transferred to the MS plain medium supplemented with hygromycin at 25 mg/l and carbenicillin without any hormone (NAA or BAP/kinetin). The frequency of regeneration varied from 7.5% -40%. Among all the six varieties, Inqulab-91showed a transformation frequency of 43.75% followed by Tartara-2000 i.e., 40% (Tables 9 & 10). More than hundred hygromycin resistant and GUS expressing plants were produced and transferred to establishment room. Plants from independent hygromycin resistant calli of all varieties were evaluated for phenotypic characterization and fertility in the glass house. About 5% (especially in Chakwal-97) of the plants were Albinos and they failed to survive in soil. All the other plants exhibited normal phenotype with a usual life cycle but could not reach to maturity. Only three plants of Tartara-2000 and four of Inqulab-91grew to maturity and produced seeds.

Establishment of transgenic lines in the glass house: Well-rooted transgenic plants grown on MS + NAA + BAP with/ without Kinetin of all the varieties as shown in Tables 9 &10 were established in the glass house.

Glass house assessment of transgenic plants of wheat: The transgenic plants were assessed for their growth by following parameters viz., plant height, length of panicle and number of seeds per panicle (Tables 9 & 10). In this study transformed wheat plants displayed statistically significant decrease in plant height and panicle length compared with control plants of all the varieties grown from seeds.

Discussion

Introduction of target genes into the genome of elite varieties and regeneration of transgenic plants with high efficiency are the most desirable strategies for production of transgenic plants in wheat. The various factors which influence transformation efficiencies are following

Hygromycin selection as a lethal dose: Hygromycin is extensively used for wheat transformation (Hauptmann *et al*., 1987). Hygromycin was used to select transformed calli from non-transformed. To determine lethal dose of hygromycin three different concentrations were tested that is 25, 50 and 75mg/l. Hygromycin at 50mg/l proved to be lethal. It has already been reported Rashid *et al*., (2001) that 50mg/l hygromycin was lethal dose for rice varieties and for Basmati rice cv. Pusa Basmati1 (Ignacimuthu *et al.*, 2006).

Selection of suitable explant for transformation: For any gene delivery system, employment of regenerable tissues as target explant is an important factor for producing the transgenic plant (Cho *et al*., 2004). A decade ago, protoplasts were used for PEG (Datta *et al*., 2000) or electroporation (Toriyama *et al*., 1988) and immature embryo for biolistics methods (Christou, 1993) to carry out wheat transformation. Later on the embryogenic calli derived from mature seed were first employed in biolistics method by Li *et al*., (1993).

Of the various explants studied embryogenic callus served as the suitable starting material and because of its efficient regeneration system its seems to be most important consideration for both *Agrobacterium* mediated (Aldemita & Hodges, 1996; Hashizume *et al*., 1999; Hiei *et al*., 1994; Lee *et al*., 1999 and Cho *et al*., 2004) and biolistics methods (Li *et al*., 1993) particularly scutellum-derived calli were shown to be served as an excellent starting material in transformation experiment, as reported for wheat (Naveed *et al*., 2009); Saad *et al*., (2004) for *Tongil* wheat by Cho *et al*., (2004).

Chan *et al*., (1993); Hiei *et al*., (1994) and Cho *et al*., (2004) described that prior to infection, pre-culture of calli in a fresh medium for four days was an important step for transformation. Pre-treatment of tissues eg., by wounding or enzymatic digestion of cell walls were also found to be essential in other studies, such pre-treatments were not needed in transforming wheat in this study as reported by Rashid *et al*., (2001) in rice.

Time of co-cultivation: Time of co-cultivation is one of the main factors among different factors affecting *Agrobacterium* mediated gene transformation. Twenty-one days old scutellum derived calli were co-cultivated with *Agrobacterium* for 1, 2, 3 and 4 days in different experiments. N_6 medium containing 2,4-D and acetosyringone with acidic pH of 5.7-5.8 used for co-cultivation of embryogenic wheat calli at 28 ± 1^{o}C. The expression of GUS 90 % was detected after two days of co-cultivation. GUS expression was decreased as co-cultivation time period was increased. These results are in confirmatory to the results of Rashid *et al*., (2001). Our results are in contrast to the results obtained by Hiei *et al*., (1994) and Mohanty (1999) in which 4 days of co-cultivation period showed highest GUS activity and with Cho *et al*., (2004) in which transient GUS expression was detected after three days of co-cultivation. The reason for this difference might be due to the difference in the wheat varieties and *Agrobacterium* strain used.

Age of calli: Age of calli is a key factor often affective in gene transformation. In the present study it was observed that 21 days old calli were appropriate for gene transfer. It was also observed that with the increase of age, the frequency of gene transformation was decreased. Hashizume *et al*., (1999) obtained high frequency of transformation i.e., 54% and 57% from 19 and 27 days old calli respectively. When 34 days old calli were used, the transformation efficiency was drastically decreased. Raja *et al*., (2009) used 21 days old scutellum derived calli and reported 18 % transformation efficiency. From these results it can be concluded that relatively younger and actively dividing cells and tissues can be used more efficiently as compared to older explants or cells in culture, for transformation studies.

Effect of acetosyringone on transformation efficiency: Acetosyringone is a phenolic compound, which is secreted at wounded site of dicotyledons. This compound enhances the *Agrobacterium*-mediated gene transformation in dicot. Monocots lack this wound

response and it was considered as the limiting factor in *Agrobacterium*-mediated gene transformation in monocots. So it was considered that its use in *Agrobacterium*-mediated transformation is indispensable. No transformation efficiency was recorded when acetosyringone was omitted from the media. The transformation was enhanced from 3.5% to 82% when acetosyringone in combination with nopaline was used in co-cultivation media. Hiei *et al*., (1994) used glucose at 10g/l with 100µM acetosyringone in the induction medium and reported a high efficiency of transgenic plant production. In the present study only acetosyringone at high concentration was shown to be required for efficient gene transformation. It was also noticed that when either of the two media i.e., basal or top media lacked acetosyringone, the calli did not show GUS activity. Maximum GUS expression (90%) was observed when acetosyringone was present in both liquid and solid media. Rashid *et al*., (2001) earlier reported that high transformation efficiency was obtained by using 50µM acetosyringone in both induction and co-culture media. The results suggested that inclusion of acetosyringone during co-cultivation was vital for wheat transformation.

Using acetosyringone to improve transformation efficiency of monocotyledonous plants including wheat is indispensable (Hiei *et al*., 1994). According to these reports, inclusion of acetosyringone is vital for wheat transformation. Acetosyringone was used at concentration ranging from 0.1 µM to 100 µM. When acetosyringone was omitted from the medium, there was no wheat callus differentiation and no transformed calli were obtained, after 14-18 days of selection calli turned brown and necrosis observed. In the case of 6 wheat varieties, without acetosyringone, no callus proliferation and differentiation were observed. It is concluded that acetosyringone played crucial role for improvement and efficient transformation which is confirmation of the findings of Hiei *et al*., (1994) that the addition of acetosyringone in co-cultivation medium induces *vir* genes, extend host range of some *Agrobacterium* strains and is essential for wheat transformation.

When 200 µM Acetosyringone was applied, 50 out of 66 calli of Inqulab-91, Chakwal-97 and Tartara-2000 showed G.I.2. Lin & Zhang (2005) optimized a new protocol for transformation of wheat. They identified a pre-culture medium (J_3) supplemented with 200 µM acetosyringone and produced high transformation efficiency of 22.2% and 23.4% respectively in two wheat varieties. They reported transformation efficiencies much higher than those (around 5%) previously reported for wheat (Aldemita & Hodges, 1996; Mohanty *et al*., 1999 and Rashid *et al*., 2001). Terada & Shimamoto, (2004) examined the concentration of acetosyringone (100-400uM) for its effect on transformation frequency at the optimal temperature of 25^{o}C with a diluted *Agrobacterium* concentration of $O.D_{600}$= 0.2. Among the acetosyringone concentrations tested in the Amino acid medium for *Agrobacterium* treatment and the $2N_6$-acetosyringone media for co-cultivation, 200 µM AS appeared to be the best concentration for stable wheat transformation. Under this concentration they were able to obtain about 70 transformants from 1g calli.

At the Acetosyringone concentration of 300 µM, size of calli increased double to the original size and variation of callus size was observed. Only Inqulab-91 showed growth and proliferation and reached up to G.I 3. All other varieties remained in G.I 2.

When Acetosyringone was applied at the concentration of about 400µM, growth and proliferation increased, size of calli of all wheat varieties increased three times to the original size. All the calli produced plants. Inqulab-91, Chakwal-97 and Tartara-2000reached up to G.I 3. These calli continued to grow on the selective medium

(Hygromycin 50mg/l). Our results are similar to Rashid *et al*., 2001 who reported in their study the resistant calli only emerged by the application of higher concentration of Acetosyringone i.e., 400µM. Our results are similar to Terada & Shimamoto, (2004) who reported that maximum number of calli showing blue color with maximum frequency of blue patches were found on those calli co-cultivated with co-cultivation medium (MS+Hygromysin and Carbinciline @ 50 µl/l) with acetosyringone at 400µM. They also reported that increase in the concentration of acetosyringone enhance the GUS activity. Bajaj *et al*., (2006) co-cultivated embryogenic ryegrass calli at a range of acetosyringone concentration of 100-800 µM. It was found that calli co-cultivated in medium containing 400µM acetosyringone showed maximum transformation events as determined by GUS assay. It is demonstrated that acetosyringone application at the concentration of 400µM was effective in the production of transgenic wheat plants.

Comparison of antibiotics in selection of transformants: In order to eliminate *Agrobacterium tumefaciens* after co-cultivation with wheat calli, the use of antibiotics in the culture medium is required (Saad *et al*., 2004). In this research study, in order to examine the bactericidal and toxic effects of carbenicillin (500 mg/l) and cefotaxime (500 mg/l) alone or in combination (cefotaxime 500 mg/l and carbenicillin 250 mg/l) on transformed calli, the transformed calli were washed in CIM1 (liq) (N_6 + 2,4-D + carbenicillin + cefotaxime + Hyg and then were shifted to CIM2 (solid) (N_6 + 2,4-D + carbenicillin + cefotaxime + Hyg. In all of the selection media 500mg/l of carbenicillin or 500mg/l cefotaxime or cefotaxime @ 500 mg/l and carbenicillin @ 250 mg/l was added in separate experiments. The calli growth was statistically low in cultures treated with cefotaxime and carbenicillin (500mg/l) and significantly increased when both the antibiotics were used in combination. Only slight necrosis (3%) in cultures was shown in the presence of cefotaxime 500 mg/l and 250mg/l carbenicillin. These results demonstrated that, carbenicillin have better inhibitory effect on bacterial growth as compared to cefotaxime and carbenicillin is the selected antibiotic in *Agrobacterium*-mediated transformation in wheat.

The first result is in contrast to earlier studies in which it was confirmed that *A. tumefaciens* cells were more sensitive to cefotaxime than carbenicillin, when explants of tobacco were incubated with these two antibiotics in two separate experiments (Lin *et al*., 1995). The differences in results can be the attributed to the use of different explants of two different plants in these studies.

Carbenicillin is the selected antibiotic in *Agrobacterium*-mediated transformation in wheat is confirmatory to results of Pollock *et al*., (1983); Okkels & Pedersen, (1988); Koncz *et al*., (1992); Lin *et al*., (1995) and Toki (2006). No toxic effects were observed by incubating tobacco leaf explants on the MS plates containing 1000µg/ml of carbenicillin alone (Lin *et al*., 1995). Barette & Cassells (1994) reported that the concentration of antibiotics required in many cases inhibit regeneration of the plant tissues. Rashid *et al*., (2001) reported that the ability of carbenicillin and cefotaxime in controlling the growth of *Agrobacterium* during the regeneration of the calli of wheat showed that there was strong inhibition of regeneration potential. The reduced regeneration capacity is in agreement with the results obtained by Lin *et al*., (1995), who reported that 500mg/l cefotaxime and 1000mg/l carbenicillin played an inhibitory effect on regeneration of *Nicotiana tobacum*. However, a positive effect of concentration up to 1000mg/l was seen in some plants (Bajaj *et al*., 2006). Barrett & Cassells (1994) found no influence of 500mg/l cefotaxime on regeneration of *Pelargonium domesticum* cv.

Grand Slam. Some investigators have ascribed the low success rate of wheat transformation to the possible toxicity to callus growth by the antibiotics (Rashid *et al*., 2001) but results of this research confirm the finding of Lin and Zhang (2005) who reported that neither of these two antibiotics had adverse effects on growth and differentiation of the calli. They tested it for most wheat varieties at the concentrations frequently applied in transformation experiments. This suggests that application of the antibiotics does not adversely effects the low efficiency of wheat transformation.

Comparison of two protocols of selection: Effects of two protocols i.e. selection following pre selection and direct selection were studied. There is a significant difference between two protocols for two parameters i.e., number of resistant calli and number of calli showing growth. In the present study the frequency of clean calli significantly decreased in the first protocol as compared to the second protocol. These results are in confirmatory to what was observed by Zhang *et al*., (1999). Necrosis was observed to be 32% and 10% in cultures screened from first and second protocols of selection respectively. Lin *et al*., (1995) reported slight necrosis of tobacco leaf explant in the presence of 2000 µg/ml of carbenicillin. In their studies they demonstrated that the toxicity of antibiotics was increased with increase in the concentration of antibiotic in the presence of auxin in the media. But no toxic effects were observed by culturing explants on MS plates containing up to 1000µg/ml of carbenicillin alone. In the present study, as in the first protocol of selection, the calli were exposed to this toxic effect of antibiotics for a longer duration thus there is significant decrease of callus growth frequency as compared to observed in calli exposed to this toxic effect for a lesser duration in direct selection protocol.

Regeneration frequency of transgenic plants and their GUS expression: Regeneration of transformed calli was carried out on Regeneration medium 1-13 (MS medium supplemented with different concentrations of IAA or NAA and BAP or kinetin or both BAP and Kinetin. The regeneration of transformed calli was observed in calli selected from direct selection protocol. While zero regeneration frequency was shown by calli selected from the selection protocol in which pre selection was also performed. Rashid *et al*., (2001) obtained low regeneration frequency from calli of *Moricandia arvensis* that were selected from direct selection. While 8% transformation efficiency was exhibited by calli selected from selection protocol in which pre selection for 7 days was also given. Pre selection for a prolonged period of 10 days exhibited a high percentage of selected calli, but resulted in lower regeneration of 6%. In the present study it was presumed that although pre selection is necessary step in case of dicot for high transformation efficiency but this step significantly decreased the transformation efficiency in case of monocots (Raja *et al.,* 2008).

High transformation efficiency was observed in Chakwal-97 i.e., 43.75%. While Inqulab-91 exhibited only 7.5% transformation efficiency. On the other hand Inqulab-91 exhibited a highest transformation efficiency of 42.5 %, which was higher than Saad *et al*., (2004), who reported Inqulab-91 with low transformation efficiency i.e., 4.8% which was probably due to adverse affect of *Agrobacterium* infection but in this study there was no adverse affect of *Agrobacterium* infection as reported by Tu *et al*., (1996).

Table 1. A general response of calli of wheat (*Triticum aestivum* L.) to different concentrations of hygromycin.

Concentration of hyg (mg/l)	Calli showing browning (%)	Percentage of calli showing necrosis (%)	Percentage of calli showing growth (%)	Response
0	0	0	80	Well proliferating
25	25	0	60	Average proliferating
50	78	22	0	Growth stop, calli become dead
75	0	98	0	Calli completely died
100	0	100	0	Calli completely died

Total calli cultured = 50

Table 2. Effect of age of calli on GUS expression after two days of co-cultivation with *Agrobacterium tumefaciens* strain EHA101 (pTCL5) in wheat (*Triticum aestivum* L.).

Wheat varieties	Average no. of gus^+ calli co-cultivated at the age of				Mean
	14 days	21 days	28 days	35 days	
Chakwal-97	8.00 d	15.00 b	5.00 de	0.00 e	7.00 b
Inqulab-91	9.00 cd	16.00 b	7.00 d	0.00 e	8.00 b
Tartara-2000	8.00 d	15.00 b	5.00 de	0.00 e	7.00 b
Mean of treatment	8.34b*	15.333 a*	5.66 c*	0.000 d*	

LSD 0.01 for variety =2.870
LSD 0.01for treatment = 1.913
LSD 0.01for variety x treatment interaction = 5.748
The data is average of three replicates
**Probability was checked by using t-test at 0.01 level of significance different alphabetic keys notify significant difference

Table 3. Effect of time of co-cultivation period with *Agrobacterium tumefaciens* strain EHA101 (pTCL5) GUS expression in wheat (*Triticum aestivum* L.).

Wheat varieties	Average no. of gus^+ calli co-cultivated for				Mean of each variety
	1 day	2 days	3 days	4 days	
Chakwal-97	0.00	8.00	2.00	0.00	2.50
Inqulab-91	1.00	6.00	1.00	0.00	2.00
Tartara-2000	2.00	8.00	3.00	0.00	3.25
Mean of each treatment	1.00 b*	7.33a*	2.00 b*	0.00 c*	

LSD 0.01 for variety =1.046
The data is average of three replicates
**Probability was checked by using t-test at 0.01 level of significance different alphabetic keys notify significant difference
NS – Not significant

Table 4. Effect of acetosyringone concentration of 400 µM on GUS expression after two days of co-cultivation with *A. tumefaciens* strain EHA101 (pTCL5) in wheat (*Triticum aestivum* L.).

Liquid medium	Bottom medium	Average number of calli showing gus expression	Average percentage gus expression (%)
+	+	18	90
+	-	0	0
-	+	0	0
-	-	0	0

Total calli tested for GUS = 20
Data is the average of three replicates

Table 5. Growth index (G.I) indicated and evaluated the degree of differentiation of callus after 15-21 days of selection on N_6 medium supplemented with 2,4-D 2mg/l, hygromycin at 50mg/l.

Varieties	Acetosyringone concentration									
	50 µM		100 µM		200 µM		300 µM		400 µM	
	A.I.G.I	C.P	A.I.G.I	C.P	A.I.G.I	C.P	A.I.G.I	C.P	A.I.G.I	C.P
Chakwal-97	G.I.0	0	G.I.1	3	G.I.2	11	G.I.2	13	G.I.3	15
Inqulab-91	G.I.0	0	G.I.0	0	G.I.1	4	G.I.2	11	G.I.2	10
Tartara-2000	G.I.0	0	G.I.1	3	G.I.0	0	G.I.2	9	G.I.3	16

AIGI = *Agrobacterium* inoculation growth index
CP = No of Calli proliferated and growth
No. of calli inoculated = 20

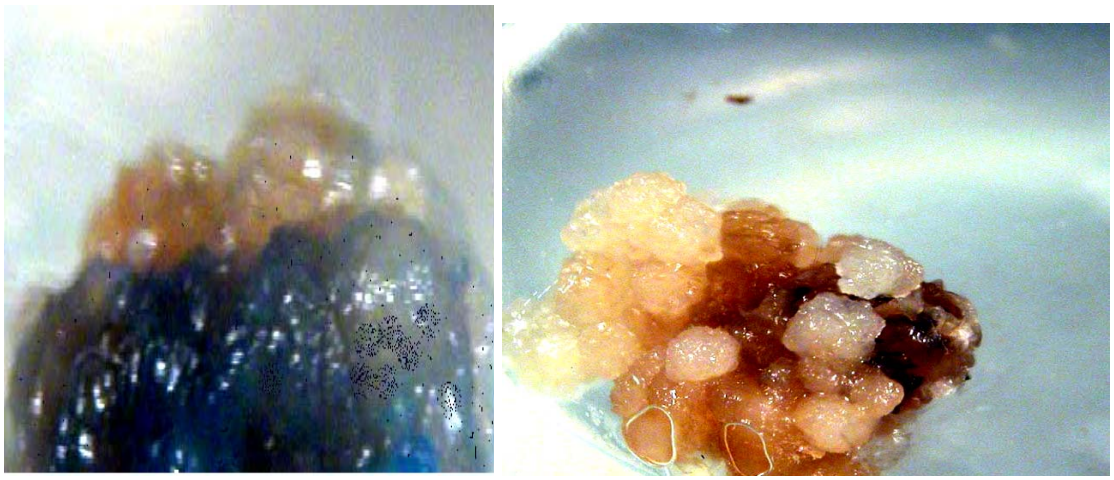

Fig. 5. A general GUS expression in wheat calli at the acetosyringone concentration of a) 200 µM, b) 400 µM

(a) (b) (c)

Fig. 6. Transient/Stable GUS expression;
a. In twenty one days old scutellum derived calli after two days of co-cultivation (Transient GUS),
b. Transformed leaf (Stable GUS)
c. Transformed callus (Stable GUS)

Table 6. Effect of cefotaxime (500 mg/l) or carbenicillin (500 mg/l) or both on the growth of calli in wheat (*Triticum aestivum* L.).

Varieties	Average no. of calli on cefotaxime 500 mg/l	Average no. of calli on carbencillin 500 mg/l	Average no. of calli on cefotaxime 500 mg/l and carbencillin 250mg/l	Mean of each variety
Chakwal-97	4.00efgh	4.00 efgh	7.00 cdef	5.00 c
Inqulab-91	4.00efgh	12.00 abc	15.00 a	10.00b
Tartara-2000	16.00a	8.00 cdef	11.00 abcd	11.66 ab
Mean of each treatment	8.00c**	8.00 c**	11.00a**	

LSD 0.01 for variety =3.214
LSD 0.01 for treatment = 1.855
LSD 0.01 for variety x treatment interaction = 5.566
The data is average of three replicates
** Probability was checked by using t-test at 0.01 level of significance different alphabetic keys notify significant difference

Table 7. Comparison of indirect and direct selection protocol in wheat (*Triticum aestivum* L.).

Variety	Indirect		Direct		Mean of each variety
	No. of resistant calli	No. of calli showing growth	No. of resistant calli	No. of calli showing growth	
Chakwal-97	9.00	5.00	13.00	9.00	9.00 cd
Inqulab-91	12.00	6.00	15.00	13.00	11.50 abc
Tartara-2000	9.00	5.00	12.00	8.00	8.50cd
Mean of each treatment	10.00 b**	5.33 c**	13.33 a**	10.00b**	

LSD 0.01 for variety =3.199
LSD 0.01for treatment = 1.847
The data is average of three replicates
** Probability was checked by using t-test at 0.01 level of significance Different alphabetic keys notify significant difference

Table 8. Transformation efficiency (T.E. %) by *Agrobacterium* in three wheat varieties

Wheat varieties	Rm used mg/l (ms + sucrose+ sorbitol + casine hydrolysate +naa + bap + kinetin	Number of scutellum -derived calli			
		No. of HygR calli produced	No. of HygR plants produced	No. of HygR and GUS + plants B	T.F. % (B/Ax100)
Chakwal-97	NAA1.0: BAP 2.0	32	21	6	7.5
Inqulab-91	NAA1.0: BAP 0.5	58	26	14	17.5
Tartara-2000	NAA1.0: Kin 0.5	55	45	32	40

No. of calli co-cultivated (A) = 80

Table 9. Glasshouse assessment of transgenic wheat plants.

Variety	Height (cm)		Length of panicle‡		No of grains per panicle‡		Means of varieties
	Control	Transgenic	Control	Transgenic	Control	Transgenic	
Chakwal-97	118.00efgh	99.00 jklm	13.00 pq	NP**	101.00 jklm	NP**	75.83 d
Inqulab-91	125.00cdef	71.00 b-g	16.00 pq	NP**	138.00 b	NP**	92.83 ab
Tartara-2000	130.00bcde	72.00bcde	18.00opq	NP**	98.00 klm	NP**	81.50 c
Means of treatments	125.1 a *	93.0 b*	16.74 d*	15.0 d*	99.62 c*	89.5 c*	

LSD 0.01 for variety= 5.253
LSD 0.01 treatment= 4.284
LSD 0.01for variety x treatment interaction = 12.87
† Values are expressed as mean
‡ Each value is mean of 5 plants (at least 5 different panicles of Chakwal-97 and Inqulab-91were examined), n=5. For each wheat line, the same 5 plants were analyzed
Values followed by the same letters are not significantly different at α=0.01
The data is average of three replicates
* Significant differences at P = 0.01 level between control and transgenic plants in the same column
**NP = not produced
***NT=not tested

Table 10. Media used for transformation protocol and regeneration of transformed calli.

Stage	Media	Composition
Callus induction	CI	MS salts and vitamins (Chu *et al*., 1975), 30 g/l sucrose, 3 mg/l 2,4-D, 4g/l gelrite, pH 5.7- 5.8
	CIM1 (liq.) + As	MS salts and vitamins, 2 mg/l 2,4-D, 50 -400µM acetosyringone, pH 5.7-5.8.
Co-cultivation	AA + As	AA media (Toriyama & Hinata, 1985), 20 g/l sucrose, 3mg/l 2,4-D, 50-400 µM acetosyringone, pH 5.7-5.8.
	CIM2 (solid) + As	CIM1, 4g/l gelrite, pH 5.7-5.8.
Pre-selection	CIM1 + Cb and Cf	MS salts and vitamins, 3 mg/l 2,4-D, 500mg/l Cb, 250mg/l Cf, pH 5.7-5.8.
	CIM2 + Cb and Cf	MS salts and vitamins + 3 mg/l 2,4-D, 4mg/l gelrite, 500mg/l Cb, 250mg/l Cf, pH 5.7-5.8.
Selection	CIM1 (liq) + Hyg Cb and Cf	MS salts and vitamins, 3 mg/l 2,4-D + 50mg/l Hyg + 500mg/l Cb, 250mg/l Cf,, pH 5.7-5.8.
	CIM2 (solid) + Hyg Cb and Cf	MS salts and vitamins, 3 mg/l 2,4-D, 50mg/l Hyg, 500mg/l Cb, 250mg/l Cf, 4g/l gelrite, pH 5.7-5.8.
Regeneration	RM - Hyg Cb and Cf	MS salts and vitamins, 3 % sucrose, 3 % sorbitol, 1 mg/l IAA, 0.5-5 mg/l BAP, 4g/l gelrite, 50mg/l Hyg, 500mg/l Cb, 250mg/l Cf, pH 5.7-5.8.
	RM2- Hyg Cb and Cf	MS salts and vitamins, 3% sucrose, 4g/l gelrite, 50mg/l Hyg, 500mg/l Cb, 250mg/l Cf, pH 5.7-5.8.

Abbreviations used

CI: Callus Induction
CIM: Callus Induction Medium
As: Acetosyringone
Cb: Canamycin
Cf: Cefataxmine
Hyg: Hygromycin
RM: Regeneration Medium

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