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RESEARCH PAPER

Optimization of factors affecting callus-based gene transformation by *Agrobacterium tumefaciens* in wheat (*Triticum aestivum* L.)

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Key Message: This research optimizes key factors influencing callus-based gene transformation by *Agrobacterium tumefaciens* in wheat (*Triticum aestivum* L.). The study identified optimal conditions including a 50 mg/L hygromycin dose, an *Agrobacterium* optical density of 0.8, 300 µM acetosyringone, 5-minute infection period and 48-hour cocultivation duration for maximizing gene transfer.

Abstract

Callus based gene transformation is a method used to modify and improve agronomic traits of crops. There are various factors which affect the gene transformation process. The most important factors are cultivar, age of callus, growth regulator, optical density of *Agrobacterium* culture, acetosyringone concentration, infection and cocultivation time, cefotaxime and hygromycin concentration etc in pre-selection and selection media. Therefore, for gene transformation of each crop, these factors must be optimized for successful and maximum gene transformation. This study was planned to optimize these factors for transformation of *DREB1A* gene via *Agrobacterium* EHA101 strain, and plasmid containing hygromycin resistance gene. The calli of four wheat cultivars were used. The results showed that 50 mg/L of hygromycin was optimized as a lethal dose for selection of transformed calli. The other parameters like optical density 0.8, and 300 µM of acetosyrinogne were optimized to enhance the process of infection and transfer of gene. 5 minutes infection time of *Agrobacterium* to wheat calli and 48-hour cocultivation time was optimized for each cultivar of wheat. 500 mg/L cefotaxime was optimized to eliminate *Agrobacterium* after cocultivation. Overall, the callus of Lasani-08 showed maximum transformation out of four wheat cultivars based on hyrgromycin resistance. This optimized protocol will be helpful in future for transfer of other genes in wheat cultivars.

Keywords: Callus, Cefotaxime, Cocultivation, Hygromycin, Infection, Selection, Transformation, Wheat

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Introduction

Wheat (*Triticum aestivum*) is a staple food of Pakistan and many other countries of the world (Khan et al., 2016; Anser et al., 2018). There are different environmental constraints that affect its production. Agriculture land is declining drastically due to urbanization and land occupied by housing societies. The agriculture sector is facing problems in terms of the high cost of fertilizer, fuel and seed etc., others include biotic and abiotic factors (Shafqat et al., 2019; Mehmood et al., 2020). High population growth rate and low yield create a gap between demand and supply. There is a need that traits of wheat to be improved by genetic engineering rather than conventional methods. One of the basic methods is callus-based transformation for gene transfer. The first successful transformation in wheat was reported in 1992 (Vasil et al., 1992) Later on, the first successful and stable transformation in wheat was reported by Cheng et al. (1997) where transgene was expressed and inherited in the next generation. Some others report in which DNA delivery was made via 'particle bombardment' and *Agrobacterium tumefaciens* in shoot apical meristem of rice (Supertana et al., 2005) and wheat (Supartana et al., 2006; Bo et al., 2008). A reproducible Agrobacterium-mediated transformation system was developed for the spring wheat cv. 'Fielder' (Hayta et al., 2019).

Various factors influence the *Agrobacterium tumefaciens*-mediated transformation (Cheng et al., 2004; Shah et al., 2016). These factors include selection of genotype, explants, *Agrobacterium tumefaciens* strain, binary vector, selectable marker gene, promoter, inoculation and co-culture conditions, callus induction and regeneration medium (Shrawat & Lorz, 2006; Jan et al., 2018). A callus-based *Agrobacterium tumefaciens* mediated transformation protocol was reported for transformation of *Xa21* gene in three wheat varieties, 50 mg/L hygromycin was used in selection medium and (200-400 µM)

acetosyringone was used to boost the gene delivery process of *Agrobacterium tumefaciens* (Rashid et al., 2010; Raja et al., 2010). Acetosyringone is a phenolic inducer of virulence (Vir) genes of *Agrobacterium tumefaciens* (Fortin et al., 1992). In transformed *Agrobacterium tumefaciens* vir gene is replaced with gene of interest. In dicots, acetosyringone is released by wounded plants cells but in monocots it is absent. It enhances the transformation rate of gene of interest in host plant tissues. Its different optimum concentration (50 µM, 100 µM, and 200-400 µM) were reported for maximum transformation (Rashid et al., 2010; Raja et al., 2010; Rashid et al., 2012). Similarly optical density 0.05 and 0.75 of *Agrobacterium* culture was reported to be optimum for successful transformation. At optical density above this level, contamination could not be handled (Rashid et al., 2010). Therefore, this study was planned to optimize the different factors for maximum transformation of gene in calluses of selected wheat cultivars.

Materials and Methods

Culturing of *Agrobacterium*

An *Agrobacterium* colony (Fig. 1) was inoculated in Yeast extract peptone (YEP) media supplemented with Kanamycine (50 mg/L) as a bacteria selectable marker and cultured overnight in a dark, at 28 °C on a rotary shaker at 105-110 rpm.

Optimization of lethal dose of hygromycin

Construct had hygromycin resistant gene (a plant selectable marker) therefore different lethal dose (0, 10, 30, 50, 70, 90 and 100 mg/L) of hygromycin was used to determine its lethal dose on non-transformed calli. These concentrations of hygromycine were supplemented in already autoclaved MS salts and vitamins media. Before autoclaving, its pH was adjusted in between (5.75 to 5.8). These already autoclaved MS salts and vitamins medium were supplemented with 30 % sucrose and 0.8% agar (gelling agent). Callus culture, which was 21 days old, was used for selection. Callus culture was incubated at 25 °C in growth room (Fig. 2). Data was recorded after 2 to 3 weeks.

Optimization of *Agrobacterium* optical density and acetosyringone

Agrobacterium culture was centrifuged at 3000 rpm for 10 minutes. The pellet (Fig. 3) of *Agrobacterium* culture, each of having optical density 0.5, 0.8, 1.1 and 1.4 at 600 nm were used in MS callus induction liquid media supplemented with four level (100, 200, 300 and 400) µM of Acetosyringone separately. Data was recorded on the basis of hygromycin selected calli.

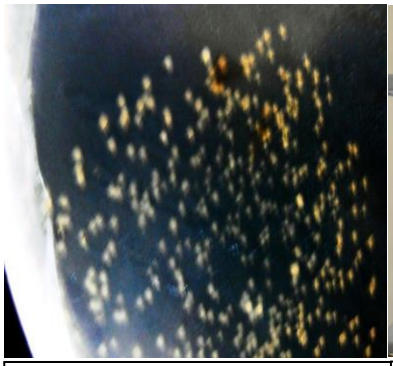

Fig.1 *Agrobacterium* colonies

Fig. 2 Calli on selection media

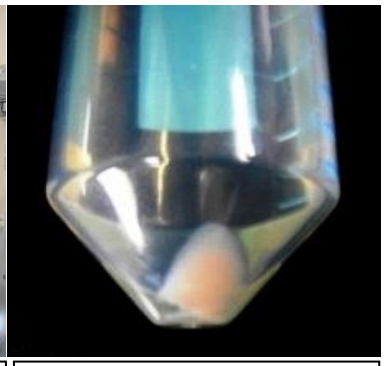

Fig. 3 *Agrobacterium* pellet

Optimization of infection and cocultivation time

The calli of four cultivars of wheat (lasani-08, GA-02, Chakwal-97and Inqlab-91) were used for this experiment to optimize infection and cocultivation time for maximum transformation. The proliferated and healthy maintained calli from maintenance media were transferred in petri plates containing *Agrobacterium* suspension with O.D. of (0.8) (optimized) and Acetosyringone (300 uM) (optimized). Three treatments i.e. 1, 5 and 10 minutes (infection time) were used to optimize the suitable infection time. Extra *Agrobacterium* was removed by blotting on sterilized filter paper after the infection process. Dried calli were shifted on patri plates having cocultivation media [MS salts plus vitamins (4.3 g/L), Sucrose (30 g/L), Myo-inositol (0.1 g/L), pH (5.75-5.80), agar (6 g/L) supplemented with Acetosyringone (300 µM)]. These cocultivation plates were placed in a dark for 48 and 72 hours at 28 °C for cocultivation.

Optimization of cefotaxime concentration

The calli were disinfected with cefotaxime after cocultivation, with its different concentrations (250, 500 and 750 mg/L) mixed with different media i.e. it was mixed only in washing media (media 1), pre-selection media (media 2) and both in

washing and pre-selection media (media 3). The data regarding elimination rate of *Agrobacterium* was recorded by applying the following formula:

$$\text{Elimination of Agrobacterium (\%)} = \frac{\text{(No. of calli having no Agrobacterium growth)}}{\text{(Total number of calli used)}} \times 100$$

Statistical analysis

Data was recorded in percentage and statistical analysis was carried out applying ANOVA with two factorial CRD using statistics 8.1. Three replications were used for each treatment. The significance of treatment means was further analyzed using LSD test.

Results and Discussion

Optimization of lethal dose of hygromycin

The lethal dose of hygromycin was optimized by using non-transformed calli on callus induction media. The results indicated the significant difference ($P<0.05$) among all levels of hygromycin in terms of calli browning ($P=0.0000$), calli necroses ($P=0.0000$) and calli growth ($P=0.0000$) (Table 1 and 2). The data in Table 3 showed that browning percentage of calli increased with increasing concentration from 10 mg/L to 50 mg/L of hygromycin, necrosis of calli increased from 30 mg/L to onward while growth percentage decreased with increasing its concentration. The data also indicated that at 50 mg/L of hygromycin, 50.83% calli showed necrosis and no growth and proliferation was observed at this concentration. So, this concentration was considered as a lethal dose and effective dose for selection of transformed calli from not transformed. While at higher concentration of hygromycin, growth of calli retarded and complete necroses were seen after two to three weeks.

Hygromycin phosphotransferase (*hpt*) gene of *E. coli* as reported by Waldron et al. (1985) showed resistance to antibiotic hygromycin B in prokaryotic cells. Hygromycin antibiotic had effects on ribosomes present in mitochondria and chloroplast of plant cells and blocked the synthesis of protein. As a result, plants showed chlorosis due to lack of chlorophyll synthesis (Benveniste & Davies, 1973). It was also widely used for selection of transgenic wheat (Hauptmann et al., 1987) and barley (Orman-Ligeza et al., 2020). The construct used in this study contained hygromycin resistant gene (plant selectable marker gene) along with *DREB1A* gene (drought tolerance gene) so transformed calli showed resistance on selection media. Therefore, hygromycin concentration (50 mg/L) was found to be a lethal dose for non-transformed calli. Thus, it was considered as an important differentiation agent for transformed and non- transformed cells of calli. Our optimized lethal dose of hygromycin for wheat calli were supported by the results of (Rashid et al., 2001; Raja et al., 2010; Rashid et al., 2010) but contradicted to some other reports such as Chen and Punja (2002) optimized 100 mg/L for selection of American ginseng transgenic plants. These variations in concentration of hygromycin for selection of transgenic calli or plants were due to sensitivity of tissues of respective plants. Transformed calli showed growth at this optimized concentration of hygromycin (50 mg/L), while non-transformed calli did not survive at this concentration. Hence 50 mg/L of hygromycin was proved to be lethal for non-transformed calli.

Table 1 Effects of hygromycin on calli browning

Source	DF	SS	MS	F	P
Hygromycine	6	30395.2	5065.87	399	0.0000
Error	77	977.1	12.69		
Total	83	31372.3			
Grand mean	20.179	CV 17.65			

Table 2 Effect of hygromycin on death of calli

Source	DF	SS	MS	F	P
Hygromycine	6	128445	21407.4	1314	0.0000
Error	77	1254	16.3		
Total	83	129699			
Grand mean	50.119	CV 8.05			

Table 3 Effect of hygomycine on wheat calli

Concentration of Hygromycin	Calli showing browing (%)	Calli showing necrosis (%)	Calli showing growth (%)
0	0^{e}	0^{e}	100^{a}
10	22.08^{d}	0^{e}	77.5^{b}
30	38.75^{b}	31.25^{d}	27.91^{c}
50	49.16^{a}	50.83^{c}	0^{d}
70	31.25^{c}	68.75^{b}	0^{d}
90	0^{e}	100^{a}	0^{d}
100	0^{e}	100^{a}	0^{d}

Optimization of acetosyringone concentration and optical density of *Agrobacterium* for transformation

The data in Table 4 showed the significant differences (P<0.05) among concentration of acetosyringone (P=0.0000) and optical density of *Agrobacterium* (P=0.0000) but there was no significant difference (P=0.1468) in interaction of acetosyringone concentrations and optical density of *Agrobacterium*. Data in Table 5 showed that when 300 µM acetosyringone concentration was used in inoculation and cocultivation media, 9.84 percent (mean value) calli survived on selection media but when concentration of acetosyringone increased (400 µM) then resistance in calli on selection media became low (7.5%). Similarly at lower concentrations (100, 200 µM) of acetosyringone, hygromcyin resistant calli were recorded 2.5 and 5.63 percent, respectively.

Acetosyringone (phenolic compound) released from wounded site of dicotyledons plants which triggers the *virA* gene of Ti plasmid in *Agrobacterium tumefaciens* that increased the efficiency of *Agrobacterium* mediated transformation. While acetosyringone in monocots was not released in response of wounds, so it was considered as a limiting factor for transformation in monocots through *Agrobacterium*. It was proved by Orman-Ligeza et al. (2020) that it enhanced transformation in barley similarly reported that acetosyringone was essential requirement of *Agrobacterium* mediated transformation in monocots. They reported that when acetosyringone was omitted from cocultivation media, no transformation was recorded while 3.5 percent to 82 percent transformation was recorded when this was included with combination of nopaline in cocultivation media (Veluthambi et al., 1989).

The results of this study in term of optimized concentration 300 µM of acetosyrinogne was in close to the results of optimized concentration of acetosyringone such as 350 µM (Tripathi et al., 2010); 200-400 µM (Raja et al., 2010) in their experiments. While contradicted to the results of optimized concentration of acetosyringone i.e., 100 µM (Ke et al., 2002; Rashid et al., 2010); 200 µM (Amoah et al., 2001); 400 µM (Toriyama et al., 1988; Weir et al., 2001; He et al., 2010; Rashid et al., 2012) which were optimized to get maximum transformation.

In this study, the results (Table 5) show that at an optical density (0.8) for *Agrobacterium*, 8.75 percent (Mean value) calli were hygromycin resistant on selection media. While at optical density (0.5, 1.1 and 1.4), the hygromycin resistant calli (5.78, 6.41 and 4.53%) were recorded, respectively. Results of this study in term of optical density closed and similar with the results of (Khanna and Daggard, 2003; Jones et al., 2005) that optimized the optical density 0.8 to 1.0 but contradicted to the results of optimized optical density O.D.600 nm=0.5 (Rashid et al., 2010) O.D 0.5-0.6 at 600 nm (Haliloglu & Baenziger, 2003; Cheng et al., 2003; Bi et al., 2006), O.D = 1.3 (Amoah et al., 2001) O.D = 1-1.5 (Cheng et al., 1997) in their transformation experiments. These variable results might be due to the difference in *Agrobacterium* strain, promoters, explants type and genotype. Rashid et al. (2010) observed that when optical density of *Agrobacterium* increased from 0.5 up to 0.75 and 1 then bacterial contamination could not be controlled. Our results supported this statement because when optical density increased from 0.8 then transformation rate decreased due to uncontrollable bacterial growth as a result lots of calli loss.

Data shown in Table 5 that due to interaction of 300 µM acetosyringone concentrations and 0.8 optical density of *Agrobacterium*, maximum hygromycine resistant calli (13.12 percent) were recorded. So, it was concluded that values of acetosyringone concentration and optical density of *Agrobacterium* were more appropriate and optimized for transformation of *DREB1A* gene in selected wheat cultivar.

Table 4 Optimization of acetosyringone concentration and optical density of *Agrobacterium* for transformation

Source	DF	SS	MS	F	P
Acetosyringone	3	462.012	154.004	96.55	0.0000
Optical density	3	150.293	50.098	31.41	0.0000
Acetosyringone* optical density	9	22.754	2.528	1.59	0.1468
Error	48	76.563	1.595		
Total	63	711.621			
Grand mean 6.3672		CV 19.84			

Table 5 Optimization of acetosyringone concentration and optical density of *Agrobacterium* for transformation

Acetosyringone (uM)	Transformation efficiency on the basis of Hygromycine Resistant Calli (%)				Mean
	Bacterial optical density				
	0.5	0.8	1.1	1.4	
100	2.5 ^{fg}	3.75^{ef}	2.5^{fg}	1.25^{g}	2.5^{d}
200	4.37^{e}	7.5^{c}	6.25^{cd}	4.38^{e}	5.63^{c}
300	9.37^{b}	13.12^{a}	9.37^{b}	7.5^{c}	9.84^{a}
400	6.87^{c}	10.62^{b}	7.5^{c}	5.0^{de}	7.50^{b}
Mean	5.78^{b}	8.75^{a}	6.41^{b}	4.53^{c}	

Optimization of infection and cocultivation time

The data (Table 6 & 7) indicated that there were significant differences ($P<0.05$) between cocultivation time, among infection time and among wheat cultivars. When calli were shifted on selection media after cocultivation, then on the basis of hygromycin selected calli it was concluded that cocultivation for 48 hours period was more suitable than 72 hours at all levels of infection time for every cultivar. Among wheat cultivars, Lasani-08 showed maximum hygromycin resistant calli [mean value (11.33%)]. The 18.33 percent hygromycin resistant calli of Lasani-08 were recorded when calli were coinfected for 5 minutes and the cocultivated for 48 hours. The lowest hygromycin resistant calli (2.0) percent were recorded for inqlab-91 when infection time was 10 minutes with cocultivation time 72 hours. The smaller number of hygromycin resistant calli at more cocultivation and infection time was due to the overgrowth of *Agrobacterium* which could not be controlled at optimized concentration of cefotaxime. It was concluded that Lasani-08 wheat cultivar was a more suitable and superior cultivar for development of transgenic plants by using optimized infection and cocultivation time. The results of this study were supported by the results of studies of Nan et al. (2006); Qiu et al. (2007). They reported that infection time for *Agrobacterium* mediated genetic transformation was more important for maximum transformation. Similarly, Quanhua et al. (2007) reported that the contamination rate increased with increasing in infection time.

Table 6 Optimization of cocultivation and infection time on transformation

Source	DF	SS	MS	F	P
Treatments	5	808.80	161.760	102.92	0.0000
Cultivars	3	371.78	123.926	78.85	0.0000
Treatments * Cultivars	15	87.96	5.864	3.73	0.0003
Error	48	75.44	1.572		
Total	71	1343.98			
Grand mean	8.5000	CV 14.75			

Table 7 Optimization of co-cultivation and infection time on transformation efficiency on the basis of hygromycin resistance

Cocultivation time	Infection time	Lasani-08	Ga02	Chakwal-97	Inqlab-91	Mean
48 hour	1	8.97^{fg}	8.67^{fg}	5.33 ^{ijk}	4 ^{jklm}	6.74^{d}
	5	18.36^{a}	14.66^{b}	11.33^{cde}	8.67^{fg}	13.25^{a}
	10	15.33^{b}	13.33^{bc}	9.33^{efg}	8^{fgh}	11.5^{b}
72 hour	1	4.66^{jkl}	4.67^{jkl}	4.67^{jkl}	2.67^{lm}	4.16^{f}
	5	11.33^{cde}	12^{cd}	10 ^{def}	7.33^{ghi}	10.16^{c}
	10	9.33^{efg}	6 ^{hij}	3.33^{klm}	2.0^{m}	5.16^{e}
Mean		11.33^{a}	9.88^{b}	7.33^{c}	5.4^{d}	

LSD at 5 percent alpha level.

Amoah et al. (2001) reported that duration of inoculation/ cocultivation and cell density increased the rate transformation of foreign gene in wheat. In case of results of this study, with increasing the cocultivation time more than 48 hours, the transformation efficiency (hygromycin resistant calli) was decreased due to overgrowth of *Agrobacterium* on calli. The optimized coinfection time for this study was 5 minutes which contradicted the results of Roy et al. (2006) that reported the maximum transformation in tomato when leaf disc was immersed in *Agrobacterium* suspension for 3 minutes. Similarly, Wu et al. (2006) reported 10 minutes infection duration in tomato. These differences in

optimized coinfection times were due to the difference in crops and explants. In this study, Lasani-08 showed the best response for transformation as compared to other ones under studied cultivars which were evaluated for transformation on the basis of hygromycin resistant calli. Because every cultivar of wheat had their own genetic makeup and ability for transformation and regeneration such as spring wheat breeding line "Bobwhite" was reported to be having high callus induction, regeneration and transformation ability (Janakiraman et al., 2002).

Optimization of cefotaxime concentration

Table 8 showed the significant differences (P<0.05) among three levels of cefotaxime (P=0.0000), media (P=0.0000) and interaction between media and cefotaxime (P=0.0000). The data (Table 9) indicated that The *Agrobacterium* eliminated at 500 mg/L and 750 mg/L of cefotaxime when it was used in media 3 (i.e., in both washing and preselection media), but the concentration (750 mg/L) of cefotaxime showed negative and toxic effects on cells of calli. Hence it was concluded that 500 mg/L of cefotaxim was optimum concentration to eliminate *Agrobacterium* from calli when calli were washed with washing media supplemented with 500 mg/L of cefotaxime and placed calli on pre-selection media supplemented with 500 mg/L of cefotaxime after cocultivation. It was observed during this experiment that the cefotaxime concentration below than 500 mg/L i.e. at 250 mg/L, *Agrobacterium* overgrowth cannot be controlled even it was used in both media i.e., (media 3) ultimately callus growth slowed down and declined the rate of transformation due to loss of large numbers of calli. The results also indicated that if 750 mg/L of cefotaxime used in washing media or pre-selection media separately even then *Agrobacterium* could not be controlled.

It was concluded that optimized concentration of cefotaxime (500 mg/L) must be used in washing media as well as pre-selection media to effectively eliminate the *Agrobacterium*. Our results are similar with the results of previous studies (Guo et al., 2007) which reported the same concentration of cefotaxime to eliminate the *Agrobacterium* after cocultivation but were contradictory to the results of studies (Xing et al., 2008; Yenchon and Te-chato et al., 2012) where they reported 200 mg/L and 400 mg/L of cefotaxime, respectively to control the *Agrobacterium.* In another report, cefotaxime (500 mg/l) was used in combination with carbenicillin (250 mg/l) (Raja et al., 2010). This difference in results might be due to the use of different explants, crops and different strains of *Agrobacteium.*

An antibiotic (carbenicillin) was also reported in various studies to control the *Agrobacterium* growth (Pollock et al., 1983; Toki et al., 2006) in *Agrobacterium* mediated transformation experiments of wheat. These antibiotics had also some lethal effects on plant tissues, but cefotaxime had minimum effects as compared to other antibiotics (Pollock et al., 1983; Okkels & Pedersen, 1988). These had been used to inhibit the growth of *Agrobacterium tumefaciens* after cocultivation. It had effects on r RNA and broke the translation process in *Agrobacterium* and plant cells.

It was observed in this study that regeneration ability of transformed calli lower as compared to non-transformed calli which were placed in regeneration media without treatment of cefotaxime. It might be due to transformation of new genes in cells that made certain changes in mechanism of regeneration or might be due to cefotaxime effects on calli. The same response of transformed calli was reported by (Pipatpanukul et al., 2004) that calli loss their regeneration ability of wheat due to cefotaxime. But results of this study were contradicted to Barrett et al. (1997) who reported no effects of cefotaxime (500 mg/L) on regeneration ability of Grand Slam (cv. of *Pelargonium domesticum*). Similarly, Lin et al. (1995) reported that antibiotics had no effects on growth of calli as well as transformation efficiency in wheat. These contradictions in results might be due to differences in genotypes and crops.

Table 8 Optimization of cefotaxime in different media to control *Agrobacterium*

Source	DF	SS	MS	F	P
Cefotaxime	2	3803.2	1901.6	229.93	0.0000
Media	2	43039.5	21519.7	2602.09	0.0000
cefotaxime*media	4	4467.4	1116.8	135.04	0.0000
Error	27	223.4	8.3		
Total	35	51533.4			
Grand Mean 32.772		CV 8.78			

Table 9 Optimization of cefotaxime in different media to control *Agrobacterium*

Cefotaxime (mg/l)	Media 1	Media 2	Media 3	Mean	Remarks
250	5.83^{ef}	4.15^{f}	45^{b}	18.32^{c}	Healthy
500	8.32^{de}	7.50^{def}	100^{a}	38.61^{b}	Healthy
750	12.50^{c}	11.65^{cd}	100^{a}	41.83^{a}	Necrosis start
Mean	8.88^{b}	7.76^{b}	81.66^{a}		

Media 1 = Cefotaxime in washing media; Media 2 = Cefotaxime in selection media; Media 3 = Cefotaxime in washing and selection media both

Conclusion

This study concluded that 50 mg/L of hygromycin is an optimum concentration for selection of callus. The *Agrobacterium* culture containing optical density 0.8 with 300 µM acetosyrinogne in culture media, 5 minutes infection and 48 hours cocultivation time showed maxim transformation result based on hygromycin resistant calli. Cefotaxime 500 mg/L was optimized to eliminate *Agrobacterium* after cocultivation. It was also observed that transformation depends upon genotype of cultivars.

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