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EFFECT OF BACTERIAL CULTURE DENSITY AND ACETOSYRINGONE CONCENTRATION ON *AGROBACTERIUM* MEDIATED TRANSFORMATION IN WHEAT

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Abstract

In the present study high efficiency *Agrobacterium tumefaciens* mediated transformation system of wheat (*Triticum aestivum* L.) cv. Inqilab-91 was developed by exploiting bacterial culture density and acetosyringone concentration. *Agrobacterium* strain EHA101 harboring binary vector pIG121Hm, containing gene for GUS activity and hygromycin resistance was used in transformation experiments. Different optical densities (0, 0.25, 0.5, 0.75 and 1.0) of bacterial suspension and different concentrations (0, 50, 100µM) of acetosyringone were evaluated in transformation studies. Maximum transformation efficiency (4.16%) was obtained with bacterial suspension of $O.D._{600nm}$ = 0.5. Contamination could not be controlled when $O.D._{600nm}$ = 0.75 and 1 was used. In case of concentration of acetosyringone, 100µM was found to be better for transformation where transformation efficiency was 15.62%. Transformation results were confirmed with GUS analysis.

Introduction

Wheat (*Triticum* spp.) is a grass of family Poaceae that is cultivated worldwide. Wheat is the most widely grown cereal crop in the world (Zhou *et al*., 2003). It is an annual and self pollinated plant. Globally, it is the second largest cereal crop behind maize. A number of environmental factors such as temperature, moisture, soil and light intensity affect the growth and yield of wheat. Generally in Pakistan, sowing starts from October and continues up to the end of December, but the optimum sowing time is the month of November. It is harvested from April to May (Shah *et al*., 2003). Wheat is particularly useful crop in terms of human nutrition as it contains good levels of proteins and carbohydrates (The Columbia, Electronic encyclopedia, 2006).

Inqilab-91 is cultivated in 70% of irrigated area of Punjab. It was released in 1991 and was obtained by cross of two parent varieties, WL711 and CROW 'S'. It is high yielding disease resistant, lodging resistant and general purpose variety suitable for rich soils under normal and late planting (Mujahid, 2004).

Wheat breeders have been able to introduce desirable traits that increase the grain yield and minimize the crop loss. In recent years biotechnology is emerging as one of the latest tools in agricultural research and is contributing towards the development of novel methods to genetically alter and control plant development, performance and its products (Patnaik & Khurana, 2001). Protocols for regenerating whole plants from single cells or clumps of cells were first generated over three decades ago. Today, these protocols form the basis of micro-propagation technologies that are relatively simple and widely used (Wambugu & Kioime, 2001).

Ozgen *et al*., (1998) cultured immature and mature embryos of 12 common winter wheat (*Triticum aestivum*) genotypes to develop an efficient method of callus formation and plant regeneration from mature embryo culture, and to compare the responses of both embryo cultures. Rashid *et al*., (2002) investigated effects of media, growth regulators and genotype for callus induction, maintenance and regeneration in wheat (*Tritcum aestivum* L. cvs. Chakwal 86, Rawal 87). Shah *et al*., (2003) evaluated the most suitable concentration of growth regulators i.e., 2, 4-D, IAA, BAP and Kn for callus induction, its proliferation and regeneration in seed explants of wheat (*Triticum aestivum* L). Alizadeh *et al*., (2004) developed a new, simple and efficient method for multiple shoot regeneration of wheat. Turhan & Baser (2004) used five media supplemented with different concentrations of NAA and 2, 4-D growth regulators and two different mature embryo sources were tested in order to obtain the best wheat callus formation. Filippov *et al*., (2006) studied the effects of different factors on the embryogenesis and plant regeneration from mature embryos of Russian spring and winter genotypes.

Genetic transformation is the process of introducing genes into plants by methods which by-pass the sexual seed production process. Transformation of cereal crops is powerful research tool for gene discovery and function to investigate genetically controlled traits and is becoming a key element in the process of varietal improvement (Jones *et al*., 2005). Wheat was among the last of the major crops to be transformed, with the first fertile transgenic plants being reported a little over a decade ago (Vasil *et al*., 1992). Furthermore, transformation still remains more difficult for wheat being more genotype dependent and having lower efficiency. Consequently, the transformation technology for wheat is still far from routine or optimized (Shewry & Jones, 2005). Genetic engineering is of significant interest for improving productivity and grower profitability (Zhou *et al*., 2003). Successful production of transgenic wheat by microprojectile bombardment or *Agrobacterium* mediated transformation techniques has been reported by several research groups (Demeke *et al*., 1999).

Agrobacterium tumefaciens has played a major role in the development of plant genetic engineering. It accounts for about 80% transgenic plants produced so far (Wei *et al*., 2000). Plant transformation mediated by *Agrobacterium tumefaciens*, a soil plant pathogenic bacterium, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *Agrobacterium tumefaciens* has the exceptional ability to transfer particular DNA segment (T-DNA) of the tumor inducing (Ti) plasmid into the nucleus of infected cells where it is then stably integrated into host genome and transcribed, causing the crown gall disease (de la Riva *et al*., 1998).

Cheng *et al*., (1997) first reported the success of *Agrobacterium* mediated transformation in wheat using immature embryos, pre-cultured immature embryos and embryogenic calli to produce fertile transgenic plants. Brettell *et al*., (1998) transformed wheat using *Agrobacterium tumefaciens* with reporter genes *GUS* and *GFP* and transformed callus tissue were obtained by applying selection with bialaphos. McCormac *et al*., (1998) demonstrated transfer of T-DNA from *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes* to cells of wheat and barley following the inoculation of immature embryos and immature embryo-derived callus. Peters *et al*., (1999) transformed wheat (cv. Chinese Spring) tissues using *Agrobacterium tumefaciens* and a new plasmid modular vector, pMVTBP. Amoah *et al*., (2001) reported the efficient transformation of inflorescence tissue of wheat variety, using the *Agrobacterium* strain AGLI harboring the binary vector pAL156.

Sarker & Biswas (2002) used different explants such as mature and immature embryos, seeds, endosperms, leaves, shoot bases and root tips of four local wheat varieties and evaluated them for their *in vitro* callus induction and regeneration as well as *Agrobacterium* mediated genetic transformation. Wang *et al*., (2002) transformed immature embryos and embryo-derived calli from two cultivars of winter wheat, using three strains of *Agrobacterium tumefaciens*. Hu *et al*., (2003) developed *Agrobacterium* mediated transformation system with glyphosate selection for the large scale production of transgenic plants. Wu *et al*., (2003) used immature embryos from range of wheat varieties and the *Agrobacterium* strain AGL1 to investigate and optimize major T-DNA delivery and tissue culture variables. The aim of the present study was to develop high efficiency *Agrobacterium tumefaciens*-mediated transformation protocol for wheat. Successful plant regeneration from cells, organs or tissues is one of the important steps in application of biotechnology for crop improvement.

Materials and Methods

Mature embryos of wheat, *Triticum aestivum* L. cv. Inqilab-91, were used throughout this study for callus induction, regeneration and transformation. Transformation was carried out with *Agrobacterium tumefaciens* strain EHA101 containing binary vector pIG121Hm. It contains GUS gene as reporter gene and hygromycin resistance gene as selectable marker. Bacterial suspension of different optical densities (O.Ds) i.e., 0.25, 0.5, 0.75 and 1.0 at 600nm were used for co-cultivation and different concentrations of acetosyringone i.e., 0, 50 & 100µM were used at the time of co-cultivation and in the co-cultivation plates to check its effect on transformation efficiency. After 15 days of selection, embryo derived calli were incubated in X-Gluc solution containing 1mg/l 5-bromo 4-chloro 3-indolyl β-D-glucuronidase, 0.5% triton X-100, 20% methanol and 50mM Sodium phosphate buffer (pH 7.0). The reaction mixture was incubated at 37°C for 2-3 days. The calli were examined under microscope.

Results and Discussion

Effect of bacterial culture density: Bacterial cultures of different optical densities (O.Ds) i.e., 0.0, 0.25, 0.5, 0.75 and 1.0 at 600nm were used in the present study to investigate its effect on transformation efficiency of wheat cv. Inqilab-91. At $O.D._{600nm}$ = 0.0 and 0.25, none of the explant was transformed. Maximum transformation efficiency (12.5%) was observed at $O.D._{600nm}$ = 0.5. These results are supported by Hu *et al*., (2003) and McCormac *et al*., (1998) who used *Agrobacterium* cell density $O.D._{600nm}$ = 0.5 and obtained maximum transformation efficiency (4.4%). In case of $O.D._{600nm}$ = 0.75 and 1.0, the transformation efficiency was 4.07% and 0.0% respectively and excessive bacterial growth was observed at these higher levels of bacterial cultures (Table 1, Fig. 1), as a result of which explants died. Sarker & Biswas (2002) obtained maximum transformation efficiency with EHA105 having an $O.D._{600nm}$ = 0.75. Wang *et al*., (2002) obtained maximum transformation efficiency when bacterial cell density $O.D._{600nm}$ = 1.0 was used. Ke *et al*., (2002) transformed wheat and barley immature embryos with *Agrobacterium* having an $O.D._{600nm}$ = 1.5. Amoah *et al*., (2001) transformed inflorescence tissue of wheat using bacterial culture density $O.D._{600nm}$ = 2.0. All these reports are quite in contrast to present study.

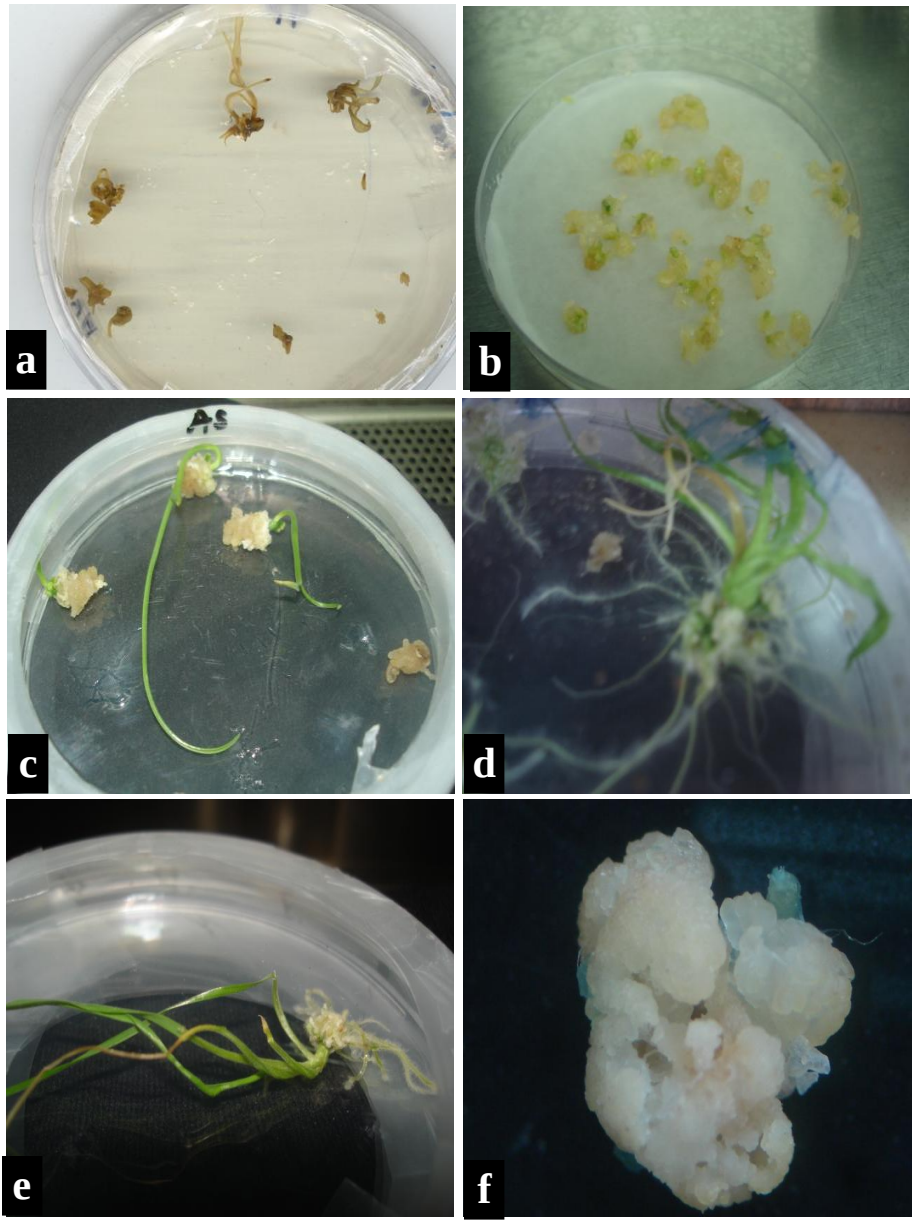

Fig. 1. Photographic presentation of *Agrobacterium tumefaciens* mediated transformation of wheat using bacterial culture density O.D.$_{600nm}$ = 0.5 and 50µM acetosyringone.
a: Untransformed mature embryos at O.D. $_{600nm}$ = 0.25
b: Co-cultivation of mature embryos with *Agrobacterium* (O.D.$_{600nm}$ = 0.5)
c: Explants on selection medium containing 50mg/l hygromycin
d: Transgenic plant originating from transformed mature embryo derived callus
e: Transgenic plant of wheat (*Triticum aestivum* L.) cv. Inqilab-91
f: GUS expression of explants.

Table 1. Effect of bacterial culture density on transformation of wheat.

BCD 600 nm	NE	SCH	G&P	PSC	CTG	GUS +ve (NBS)	TE (%)	PF
0	96	Nil	Nil	Nil	10	0	0	Nil
0.25	96	Nil	Nil	Nil	10	0	0	Nil
0.5	96	66	44	68.75	10	14	12.5	12
0.75	96	24	15	25	10	21	4.17	3
1.0	96	9	4	9.37	10	26	0	0

BCD = Bacterial culture density, NE = No. of explants, SCH = Selected calli on hygromycin, G&P = Growth and proliferation of calli, PSC = Percentage of Selected calli, CTG = Calli tested for GUS, TE = Transformation efficiency (%), PF = Plantlet formation, NBS = Number of Blue spots

Table 2. Effect of different concentrations of acetosyringone on transformation of wheat.

CA (µ M)	NE	SCH	G&P	PSC	CTG	GUS +ve (NBS)	PF	TE (%)
0	96	3	1	3.12	10	0	0	0
50	96	57	36	59.37	10	22	6	6.25
100	96	62	49	64.58	10	29	11	11.46
150	96	77	58	80.21	10	36	15	15.62
200	96	54	35	56.25	10	39	7	7.29

CA = Concentration of acetosyringone, NE = No. of explants, SCH = Selected calli on hygromycin, G&P = Growth and proliferation of calli, PSC = Percentage of Selected calli, CTG = Calli tested for GUS, NBS = Number of Blue spots, PF = Plantlet formation, TE = Transformation efficiency (%)

Effect of Acetosyringone concentration: Acetosyringone is an alcoholic compound that enhances the *Agrobacterium* infection and T-DNA delivery process. Different concentrations of Acetosyringone i.e., 0, 50, 100, 150 and 200µM were used at the time of co-cultivation and in the co-cultivation plates. No transformation was observed with 0.0µM acetosyringone. When 50µM of Acetosyringone was used, 59.37% of calli were selected on hygromycin and transformation efficiency 6.25% was observed. Transformation efficiency was increased with the increase of Acetosyringone concentration up to 150µM and maximum transformation efficiency (15.62%) was observed at 150 µM concentration (Table 2, Fig. 1). In previous studies, McCormac *et al*., (1998) and Ke *et al*., (2002) used 100µM Acetosyringone and found that presence of Acetosyringone increased the efficiency of transformation. Beyond the level of 150µM an abrupt decrease was observed in transformation efficiency and only 7.29% of the calli were transformed at Acetosyringone concentration of 200µM. our results are in contrast with Amoah *et al*., (2001) who added Acetosyringone to final concentration of 200µM and obtained increased transformation efficiency.

GUS Assay: The mature embryos after 15 days of selection were tested for transient GUS expression. In case of $O.D._{600nm}$ = 0 and 0.25, no GUS expression was observed. In case of $O.D._{600nm}$ = 0.5, 4 blue spots per callus were observed. Although the GUS expression increased when $O.D._{600nm}$ = 0.75 and 1.0 was used, but formation of transformed plants was low due to bacterial contamination. In absence of Acetosyringone, no GUS expression was observed while an increase in GUS expression was observed with the increase of Acetosyringone concentration (Fig. 1).

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