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Full Length Research Paper

Development of Regeneration and Transformation Protocol for Local Sudan's wheat (*Triticum aestivum* L) Cultivars

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The objective of this study was to optimize a simple and efficient *Agrobacterium*-mediated genetic transformation protocol for two locally grown wheat cultivars (Bohain and Elnielain) using mature embryos. Transformation was carried out with disarmed *A. tumefaciens* strain EHA105 harbouring a binary vector pCAMBIA1305.2. Conditions for *in vitro* regeneration of *Agrobacterium*-infected explants were optimized using Murashige and Skoog (MS) medium supplemented with various concentrations and/or combinations of plant growth regulators (PGRs) and Hygromycin. Results indicated that 70 and 80% of the embryogenic explants of Bohain and Elnielain, respectively, have survived when exposed to *Agrobacterium* inoculums of 0.1 O.D$_{600}$ and selection on medium containing 15 (Elnielain) or 25 (Bohain) mg/l of Hygromycin. The putative transformed explants were regenerated on MS medium containing 4.0 mg/l 2,4-D for shoot initiation, transferred to MS medium with 4.0 mg/l 2,4-D plus 1.0 mg/l Zeatin for Shoot elongation, then maintained on MS medium supplemented with a combination of 1.0 mg/l of each of 2,4-D, Zeatin and gibrellic acid (GA$_3$). This protocol resulted in transformation efficiency of 20% for Bohain and 23% for Elnielain. GUS expression was observed in transformed shoots but never in the control plants. PCR amplification of DNA extracted from the transformed tissues demonstrated the generation of the expected amplicon, corresponding to neomycin phosphotransferase II (*npt*II), hygromycin phosphotransferase II (*hpt*II), cauliflower mosaic virus-35S (CaMV-35S) promoter and nopaline synthase (NOS) terminator genes. This result strongly verifies the successful transformation of the two locally wheat cultivars and paves the way for problem solving-applications encompassing these or other Sudanese wheat cultivars of economic importance.

Keywords: wheat, regeneration, *Agrobacterium*, transformation, pCAMBIA.

INTRODUCTION

Wheat (*Triticum aestivum* L.) is a member of the family Poaceae, which includes major cereal crops of the world such as maize, millet, barely and rice (Curtis, 2002). It exceeds in acreage and production every other grain crop including rice, maize, etc. (Omara, 2001; Mitchell et al., 2005). Wheat is the first strategic cereal crop for the majority of world's populations and it is one of the most important crop for meeting the world's basic food needs (Sarkar et al., 2009).

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Sudan, one of the world's hottest countries, expanded wheat cultivation in the last decades to latitudes lower than 15°N as a winter crop, occupying the largest area in Sudanese irrigated schemes. Like all other sub-Saharan African countries, average wheat yields in Sudan are very low due to hot and dry climate (Saunders and Hettel, 1994). It was estimated that 976 metric tonnes were produced in 2010/2011 in irrigated areas compared to only 6 metric tonnes in rainfed areas; productivity in irrigated areas is estimated to be around 435.96 kg/fed compared to 261.24 kg/fed in rainfed areas (Agricultural Statistics Department Report, 2011). The effect of drought and heat constraints on yield stability have increased in importance as climate change lead to increasingly hotter and drier seasons and consequently shortage of water. Even under irrigation, the crop is commonly subjected to drought spells which may occur early in the season or terminally at grain filling and grain development (Farag, 2004).

Desirable traits have been incorporated into wheat genotypes by genetic introgression through conventional plant breeding efforts. However, such efforts are restricted by two principal constraints: absence of target genes in a compatible genotype and limited genetic variability in primary and secondary gene pools (Haussmann et al*.,* 2004). To overcome such constraints, alternative crop improvement methods such as genetic transformation could be used.

The production of transgenic plants is a complex procedure including introduction of foreign DNA into host cells, integration of the foreign DNA into the host genome, expression of the integrated genes in a controlled way and stable inheritance of the new trait (Rakszegi et al., 2001).

Of the several plant transformation methods, *Agrobacterium*-mediated plant transformation remains the method of choice in less equipped laboratories like those in the developing countries. It was believed that *Agrobacterium*-mediated plant transformation could only be used successfully with dicotyledonous plants, being natural hosts of *A.tumefaciens*; monocotyledonous plants including cereals were excluded from the range of *A.tumefaciens* hosts partly due to the demonstrated inability to produce signaling compounds for activation of *vir* genes and the lack of a typical wound response (Usami et al., 1987). Nowadays, it has been possible to transform many agronomically important cereal crops, such as maize (Thompson et al., 1995; Wang et al., 2000) and rice (Rasul et al., 1997; Chen et al., 1998; Mohanty et al., 1999) using *A. tumefaciens*. There are also some reports on *Agrobacterium*-mediated transformation in wheat (Sarker and Biswas, 2002; Patnaik et al., 2006; Jones et al., 2007).

Several factors were identified as influencing the efficiency of T-DNA delivery via *Agrobacterium*, these include: type of explant; *Agrobacterium* strain; plasmid vectors; *Agrobacterium* density; medium composition; transformation conditions such as temperature and time during pre-culture, inoculation and co-culture; surfactants or induction agents in the inoculation and co-culture; and antibiotics or selectable markers, among others (Jones et al., 2005; Bhalla et al., 2006; Opabode, 2006; Kumlehn and Hensel, 2009).

The present investigation was undertaken to optimize conditions for the development of an efficient protocol for transformation of two wheat cultivars *viz*. Elnielain and Bohain which are grown in different regions of Sudan.

MATERIALS AND METHODS

Plant material

Seeds of two popular wheat cultivars, Elnielain and Bohain, were obtained from Agricultural Research Corporation (ARC), Ministry of Agriculture, Sudan. Mature seeds were surface-sterilized with 70% (v/v) ethanol for 5 min, washed several times with sterile distilled water, immersed in 10% clorox (5.25% sodium hypochlorite) for 15 min, and washed in three changes of sterile distilled water. The seeds were then immersed in sterile water at 25-30°C for 2 hrs to imbibe water. Mature embryos were isolated from turged seeds by removing the entire axis with a scalpel under aseptic conditions.

Bacterial strain and plasmid

Agrobacterium tumefaciens EHA105 strain harbouring the pCAMBIA 1305.2 was used for transformation. The construct was under the transcriptional control of cauliflower mosaic virus 35S promoter (CaMV-35S), nopaline synthase (*Nos*) terminator, hygromycin phosphotransferase II (*hpt*II*)* and neomycin phosphotransferase II (*npt*II) (conferring Kanamycin resistance) genes as selection markers for transformed explants and bacteria, respectively and GUS-intron (uidA-β-glucuronidase) as a reporter gene. Cloning of the construct was carried out as follows: *E. coli* was transformed with a binary vector containing the *ori* and *npt*II genes following the freeze-thaw method (Burrow et al., 1990). In this method, 200µl of LB medium-based suspension of competent *E. coli* cells and 1µl of pCAMBIA1305.2 vector were added into a sterile tube. The tube was incubated on ice for 40 min, followed by a heat shock at 42°C for 2 min and re-incubation on ice for 20 min. Then, 0.5 ml of LB medium was added and the mixture was incubated in a shaking incubator at 37°C with 250 rpm for 1 hr. The bacterial solution was then spread onto a solid LB medium (in Petri plates) containing 50mg/l kanamycin for selection of transformed cells. The plates were incubated at 37°C until single colonies appeared and transformation events were confirmed by restriction enzyme digestion and PCR

Table 1 Media used for wheat transformation experiments

Medium	Constituents
Pre-culture	1mg/l ZEA in combination with 4.0 mg/l 2,4-D
Co-cultivation	PGRs-free MS medium + 300 mg/l cefotaxime + 50mg/l vancomycin
Washing	1mg/l ZEA + 250mg/l cefotaxime + 50 mg/l vancomycin
Pre-selection and shoot induction	PGRs-free MS medium
Selection	4.0mg/l 2,4-D + 1mg/l ZEA + 300mg/l Cefotaxime + 50 mg/l Vancomycin + 15mg/l Hygromycin
Shoot elongation	4.0mg/l 2, 4-D
Rooting	1mg/ 2,4-D + 1.0 mg/l GA_3+1mg/l ZEA

analysis.

The binary vector (pCAMBIA1305.2) was extracted from the transformed *E. coli* and introduced into *A. tumefaciens* strain EHA105, containing a helper plasmid by the freeze–thaw method as described previously. PCR analysis of the *npt*II gene was conducted to prove successful transformation. The transformed *Agrobacterium* cells were then used for transformation of Bohain and Elnielain wheat cultivars.

Optimization of transformation conditions

The culture media used during transformation and regeneration experiments consisted of MS medium with vitamins (Murashige and Skooge, 1962) supplemented with 30g/l Sucrose and solidified with 2.0 mg/l gelrite (except for the washing medium) and its pH was adjusted to 5.0. Different concentrations/combinations of plant growth regulators (PGRs) were added to the medium, during optimization experiments, according to the intended purpose of use (Table 1).

To facilitate the development of a protocol for *Agrobacterium*-mediated transformation of wheat mature embryos, factors previously reported to affect transformation efficiency were optimized in a series of experiments. In all experiments, completely randomized designs with ten replicates were used. Each replication consisted of ten explants in a Petri dish (100 x 15 mm) which results in a total of 100 explants per treatment.

Effect of PGRs on explants' regeneration

Different concentrations of 2,4-D (0.5,1.0, 1.5, 2.0, 3.0 and 4.0mg/l) and Zeatin (0.5, 1.0. 1.5 and 2.0mg/l) were tested to determine the optimum PGR concentration/combination to be used in the pre-culture, co-cultivation, pre-selection, selection and regeneration media.

Effect of pre-selection antibiotics on *A. tumefaciens* growth and explants survival

200µl of *Agrobacterium* suspension (OD_{600}=1) was transferred to a sterilized Petri dish and 20 ml of sterilized LB medium supplemented with different concentrations of Cefotaxime (200, 250, 300, 350, 400, 450 and 500 mg/l) alone or in combination with Vancomycine (50 and 100 mg/l) were added. After thorough mixing, the plates were incubated for 48 hours in the dark at 28°C. Colonies which appeared in the incubated plates were counted and recorded. The same concentrations were tested to determine the effective concentration of antibiotic/s that inhibits the survival of the non-transformed explants. The minimum concentration of each antibiotic that inhibited the growth of *Agrobacterium* and the non-transformed explants was used in the pre-selection medium.

Effect of hygromycin on explants survival

To screen for an appropriate hygromycin concentration for transformants selection, embryogenic calli explants were transferred to the callus induction medium supplemented with different concentrations of aqueous hygromycin B (0, 5, 10, 15, 20, 25 and 30 mg/l) in Petri dishes (09 cm in diameter) each containing 10 explants. The explants were incubated for two weeks in the dark at 25°C and then transferred to a 16/8 h (light/dark) photoperiod of 80 lmol m^{-2} s^{-1} at 25°C. The number of regenerated shoots was recorded after 4 weeks. Control explants were cultured on hygromycin-free shoot induction medium.

Effect of pre-culture and co-cultivation periods on transformation frequency

Mature embryos (*n* = 100) of each cultivar were isolated and maintained on pre-culture medium (Table 1) in the dark at 25°C for 2, 4, 6 or 8 days prior to inoculation with *Agrobacterium*. After inoculation, the infected explants were transferred to co-cultivation medium (Table 1) and incubated in the dark at 25°C for 72 hrs. Percentages of induced calli on the pre-culture medium as well as percentages of survived explants in the co-cultivation medium were recorded after 24, 48 and 72 hrs of incubation.

Table 2 Primers used for verification of transformation, their sequences, target genes, expected amplicons' sizes and PCR programs.

Target gene	Primers Sequences 5´- 3´	PCR amplification program	Expected band size (bp)
35S	GCT CCT ACA AAT GCC ATC A GAT AGT GGG ATT GTG CGT CA	Denaturation temperature: 20 s at 94°C No. of cycles: 35 Amplification steps: 2 min at 94°C, 10s at 54°C, 50s at 72°C Final extension 72°C 5min.	195 or 390
NOS	GAA TCC TGT TGC CGG TCT TG TTA TCC TAG TTT GCG CGC TA	Denaturation temperature: 20 s at 94°C No. of cycles: 35 Amplification steps: 94°C at2 min, 10 s at 54°C °, 50 s at 72°C Final extension: 5min at 72°C	180
HptII	GCGTGGATATGTCCTGCGGG CCATACAAGCCAACCACGG	Denaturation temperature: 20 s at 94°C No. of cycles: 35 Amplification steps: 94°C at2 min, 20 s at 60°C, 50 s at 72°C Final extension: 5min 72°C	598
nptII	GAG GCT ATT CGG CTA TGA CTG ATC GGG AGG GGC GAT ACC GTA	Denaturation temperature: 20 s at 94°C No. of cycles: 35 Amplification steps: 2 min at 94°C, 20s at 57°C, 50 s at 72°C Final extension: 5min at 72°C	679

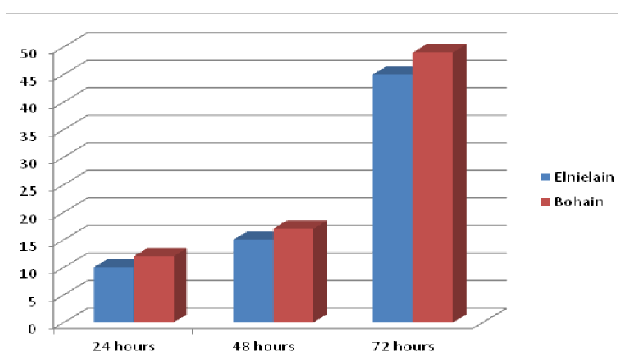

Figure 1 Percentages of callus induction from embryos of Elnielain and Bohain cultivars in MS medium supplemented with 4.0 mg/l 2,4-D

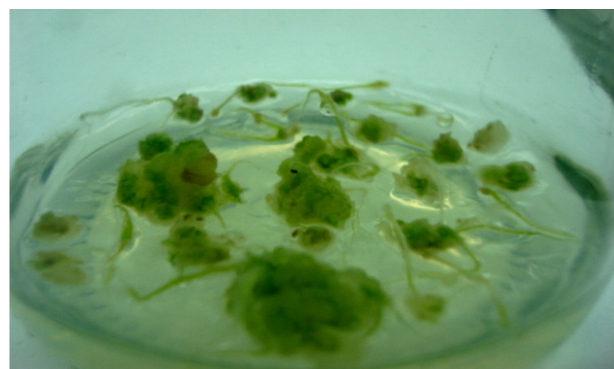

Plate 1 Induced calli from mature embryos of Bohain cultivar in MS medium supplemented with 2.0 mg/l 2,4-D

Transformation and regeneration

Wheat embryogenic explants (*n*=100) of each cultivar were cultured on Pre-culture medium for one week. Explants were then carefully submerged in *Agrobacterium* inoculum (OD_{600}=1) for 20 min with gentle swinging, dried under aseptic conditions on sterile filter papers, transferred to the co-cultivation medium and

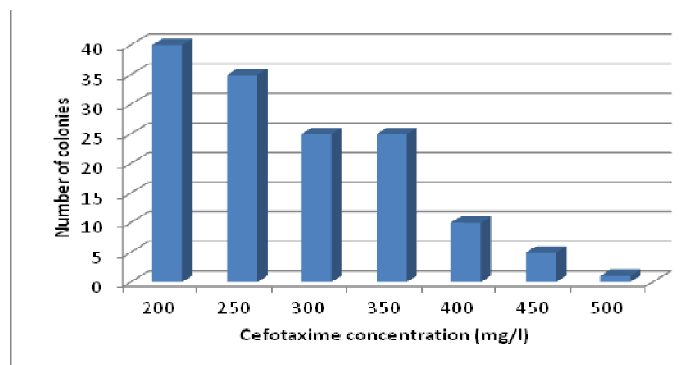

Figure 2 Effect of cefotaxime on *Agrobacterium* growth

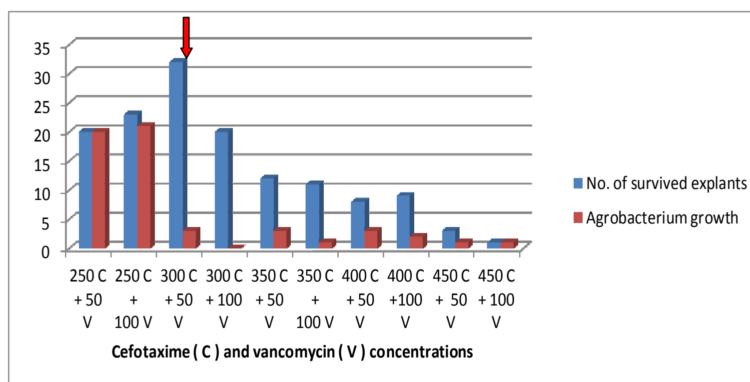

Figure 3 Effect of cefotaxime and vancomycin concentration/combination on explant survival and *Agrobacterium* growth

Table 3 Effect of Hygromycin concentration on regeneration percentage of wheat cultivars

Hygromycin concentration (mg/l)	% of survived explants	
	Elnielain	Bohain
5	40	38
10	20	23
15	0	3
20	0	1
25	0	0
30	0	0

incubated in the dark at 25°C for 3 days. Explants were then washed by washing medium, dried on sterile filter papers and transferred to plates containing MS medium supplemented with 15 (Elnielain) and 25(Bohain) mg/l hygromycin for selection. The plates were sealed with parafilm and explants were left, at 25°C with a 16h/8h (light/dark) photoperiod, in the culture room. Hygromycin-resistant calli obtained after a second round of selection were transferred to a fresh selection medium and considered as putative transformants. The fresh and healthy-looking hygromycin-resistant calli were repeatedly sub-cultured in a fresh selection medium every two weeks to avoid the formation of chimeric escapes. Survived calli were then transferred to shoot induction medium. After 4 - 6 weeks, shoots were excised and transferred to shoot elongation medium for shoot development and elongated shoots were transferred to rooting medium for root development. A set of explants which was not co-cultivated with *Agrobacterium* was also regenerated, as described above, as a negative control. Transformation frequency was expressed as a percentage of the number of shoots recovered from hygromycin relative to the total number of incubated explants.

Verification of transformants by histochemical analysis

Putative transformed explants were analyzed for GUS

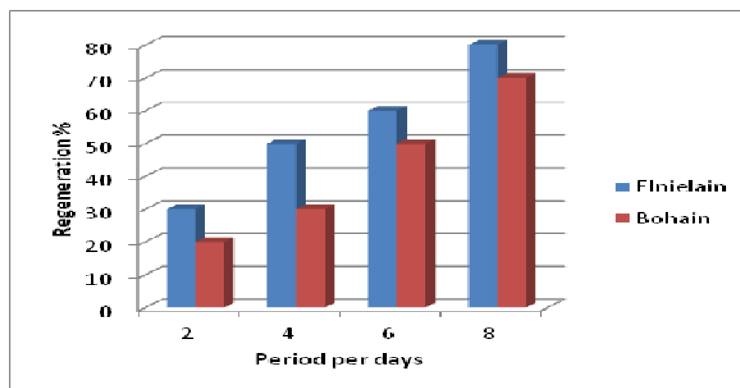

Figure 4 Effect of pre-culture period on regeneration of transformants

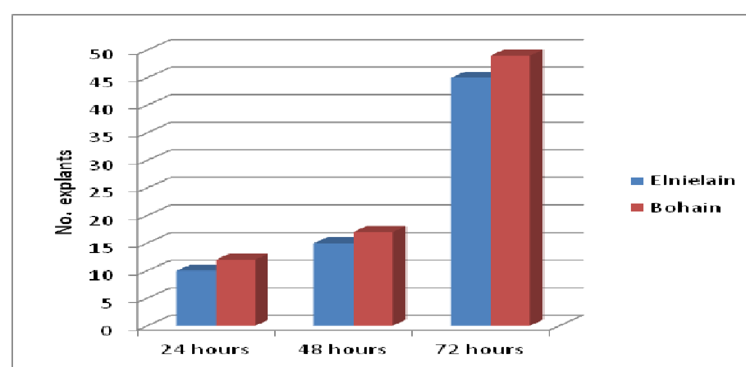

Figure 5 Effect of co-cultivation period on explants vigor

activity according to Jefferson (1987). Plant cells were incubated at 37°C for 24 hrs in GUS-staining solution (0.5 mM of X-gluc, 10 mM EDTA, 0.5 mM ferricyanide, 0.5 mM ferrocyanide, in 0.1 M phosphate buffer, pH 7.0).

Stained explants were washed in 70% ethanol and examined for GUS activity under stereomicroscope.

Molecular verification of putative transformants

Genomic and plasmid DNA extraction

Genomic DNA was extracted from leaves of putative transformed plantlets following the CTAB (cetyl trimethyl ammonium bromide) protocol described by Doyle and Doyle (1987). Plasmid DNA of *A. tumefaciens* EHA105 strain was also isolated according to the method of Sambrook and Russell (2001) and used as a positive control.

Polymerase Chain Reaction (PCR)

To confirm the presence of the inserted construct in the regenerated putative transformed plants, four primers were used to amplify four of the construct regions *viz.* The cauliflower mosaic virus 35S promoter (CaMV-35S), nopaline synthase NOS terminator, hygromycin phosphotransferase (*hpt)* and neomycin phosphotransferase II (*npt*II) genes. The primers used, their sequences, expected band size and amplification programs are presented in Table 2.

RESULTS

The best concentration of 2,4-D for induction of calli from mature embryos of both wheat cultivars was 4.0 mg/l (Figure1; Plate 1). Induced calli in this 2,4-D concentration were compact, nodular, whitish to creamy in colour and were larger than those obtained in other tested 2,4-D concentrations. These calli were then used in the subsequent transformation experiments.

Effect of pre-selection antibiotics on *A. tumefaciens* growth and explants' survival

When cefotaxime was used at lower concentrations of 200 - 350 mg/l an excessive *Agrobacterium* overgrowth was observed. Although at a higher concentration of cefotaxime (500 mg/l) satisfactory inhibitory effect on

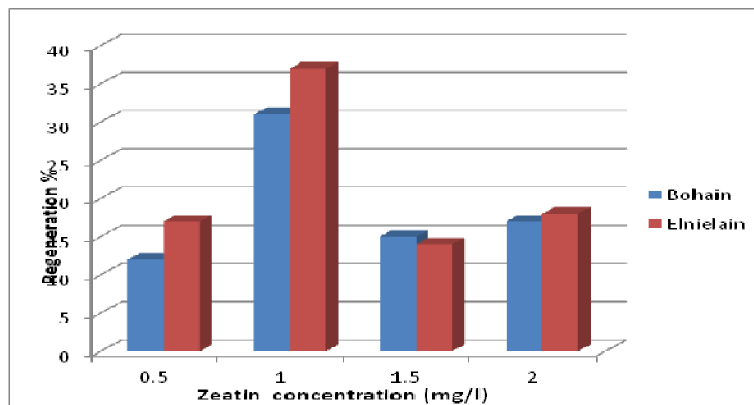

Figure 6 Regeneration % of wheat genotypes on MS medium supplemented with different concentrations of Zeatin

Plate 2 Regeneration on MS medium+ 1.0 mg/l Zeatin

Agrobacterium growth was achieved, obvious browning and death of the explants was remarkable (Figure 2). However, a combination of 300 mg/l Cefotaxime and 50 mg/l Vancomycin was found to be effective in eliminating *Agrobacterium* overgrowth without negatively affecting the explants vigour (Figure 3).

Optimization of hygromycin B concentration for calli selection

Calli initiated from mature embryos were plated on MS medium (10 calli/Petri plate) supplemented with 4.0mg/l 2,4-D and different levels of hygromycin B as a selection agent. For both cultivars, calli began to show necrotic lessions after 5 days in media containing 0, 5, 10, 15, 20, 25 or 30 mg/l hygromycin. All calli, whether derived from Elnielain or Bohain cultivars, died after 14 days of incubation in media containing 15 and 25 mg/l hygromycin, respectively (Table 3). These Hygromycin concentrations, 15 and 25 mg/l, were used each during transformation of its respective cultivar.

Pre-culture and co-cultivation periods

Pre-culture and co-cultivation periods have notable effects on the percentage of callus induction in both cultivars. When wheat embryogenic calli were pre-cultured for eight days prior to inoculation with *Agrobacterium*, 80 and 70% of Elnielain and Bohain explants have survived, respectively, and were considered as putative transformants (Figure 4). When the explants were transferred to the selective medium immediately after inoculation with *Agrobacterium* (no co-cultivation), none of them have survived. The transformation frequency was very low (10 and 12% for Elnielain and Bohain, in order) after one day co-cultivation, but increased greatly (45 and 49%, respectively) when the co-cultivation period was prolonged to three days (Figure 5).

Regeneration of putative transformed explants

Transformed explants were maintained on MS medium

Plate 3 Regenerated transgenic plant

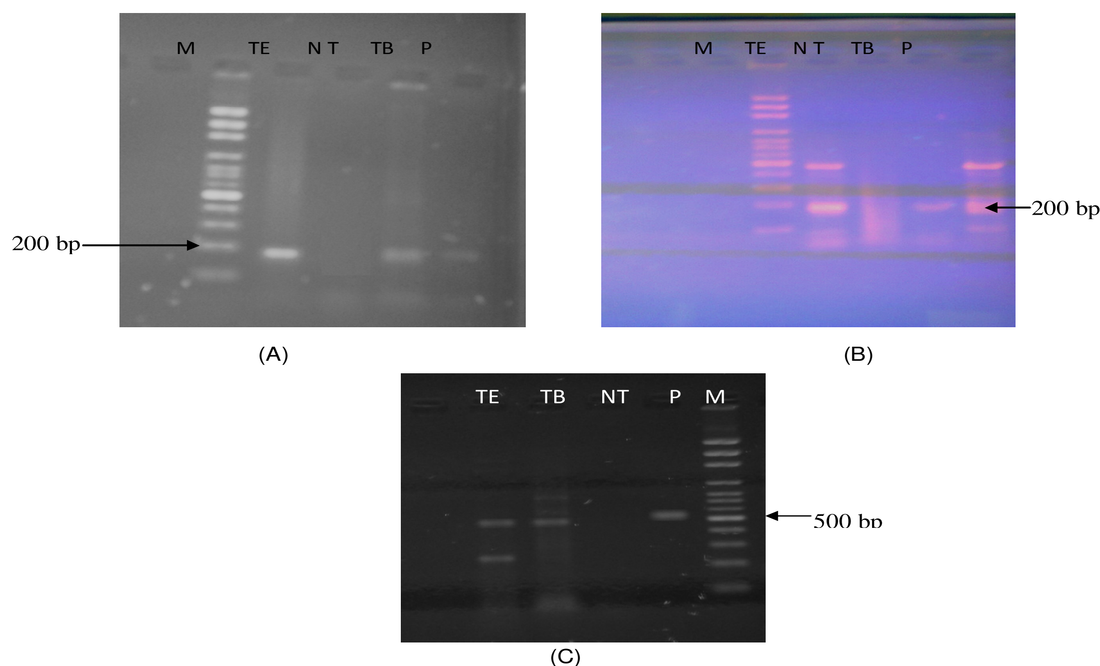

Figure 7 PCR analysis for the detection of: A) NOS terminator, B) 35-S-promoter and C) Hygromycin resistance gene in the putative transformants. TE and TB: amplification products of DNA extracted from transformed seedlings of Elnielain and Bohain cultivars, respectively; NT: PCR product of a non-transformed plantlet; P: PCR product of DNA extracted from the plasmid; M: 100 bp ladder (Invitrogen).

containing 4 mg/l 2,4-D for 17 days and then transferred to MS medium supplemented with different concentrations of Zeatin for regeneration. Results (Figure 6; Plate 2) showed that MS medium supplemented with 1 mg/l Zeatin was optimal for regeneration of putative transformants of the two cultivars under study. Long shoots were cut off and vertically inoculated into root induction medium for rooting.

Transformation efficiency

A total of 100 calli induced from mature embryos of each wheat cultivar (Elnielain and Bohain) were inoculated with *A. tumefaciens* carrying pCAMBIA1305.2 binary vectors. About 38% (Elnielain) and 40% (Bohain) of co-cultivated calli were healthy on the first selection medium containing 15 (Elnielain) or 25 (Bohain) mg/l hygromycin. When these embryogenic calli (non-embryogenic were discarded) were subjected to a second round of selection on the same medium for 6 weeks, 20 and 23% of putative transgenic explants of Elnielain and Bohain cultivars, respectively, produced shoots while the control (untransformed) explants (not co-cultivated) did not. Shoots were transferred to the rooting medium and roots formation was observed within two weeks (Plate 3).

Verification of transformants

When hygromycin-resistant calli were stained with X-Gluc, GUS activity, indicated by the presence of blue

spots, was observed in all of these explants. However, no such spots were observed in any of the non transformed (control) explants.

For further verification of transgenesis, DNA from all T_0 seedlings and from the non transformed seedling, was extracted and amplified with primers specific for the *hpt*, CaMV-35S promoter and Nos terminator genes. DNA from all putative transformants tested showed the expected target band with its respective primer while no amplification products were obtained with DNA extracted from the control plants (Figure 7), this was taken as a proof of transgenesis.

DISCUSSION

Although cereals are not natural hosts for *Agrobacterium*, many studies have been carried out worldwide to optimize conditions for successful *Agrobacterium*-mediated wheat transformation using local genotypes (Jones et al., 2005). In this study, optimum conditions for transformation and regeneration of two Sudan's locally grown wheat cultivars were determined. It was generally reported that *in vitro* regeneration can be highly influenced by different factors such as plant genotype, source of explants and plant growth regulators used in different regeneration media (Przetakiewicz et al., 2003; Rashid et al., 2009). Also, it is well established that transformed and non-transformed explants differ in their regeneration requirements (Schroeder et al., 1993).

In vitro regeneration of wheat have been successfully achieved using different explant types such as mature and immature embryos, seeds, endosperm, leaves, shoot bases and root tips (Amoah et al., 2001; Wang et al., 2007; Wu et al., 2008; Yang et al., 2008; Wang et al., 2009). In these studies, the immature embryo proved to be the best for callus induction and shoot regeneration. However, the availability of immature embryo is limited by wheat growing season or requires expensive and sophisticated growth chambers. On the other hand, mature seeds of wheat are available throughout the year, hence can be used for plant regeneration at any convenient time (Ganeshan et al., 2006). In this study, high frequency of callus induction was obtained by using mature embryo culture. Antibiotic resistance is by far the most widely used selection system in *Agrobacterium*-mediated transformation of wheat (Rashid et al., 2010). Several antibiotics have been used after co-cultivation at the selection stage to control *Agrobacterium* overgrowth. Of these, timentin (Hensel et al., 2009, Wu et al., 2009), carbenicillin (Cheng et al., 1997) and cefotaxime (Bi et al., 2006; Chugh and Khurana, 2003) were mostly used. Our results indicated that a combination of Cefotaxime (300 mg/l) and Vancomycin (50 mg/l) was optimum for controlling *Agrobacterium* overgrowth and maintaining satisfactory explants vitality. In consistence with the obtained results, Wiebke et al. (2006) reported that the use of cefotaxime plus vancomycin in the selection medium did not affect soybean somatic embryos for 63 days. In addition, it was demonstrated that the combination of cefotaxime and vancomycin stimulates organogenesis in *Pinus pinea* (Humara and Ordás, 1999) and *Prunus armeniaca* (Burgos and Alburquerque, 2003). In contradiction to the results reported here, Li et al. (2008) have shown that 500 mg/l cefotaxime did not inhibit both callus induction and shoots regeneration from cotyledon explants of *Jatropha curcas*. As cereals are more sensitive to hygromycin, kanamycin is used for selection of transformed tissues in most cereal transformation studies (Janakiraman et al., 2002). In this study, hygromycin concentrations of 15 and 25mg/l were found to be effective in selecting transformed tissues of both wheat genotypes. Similarly, Permingeat et al. (2003), Supartana et al. (2006) and Zale et al. (2009) used 25, 30 and 60 mg/l hygromycin as a selection agent, respectively.

Pre-culturing explants prior to inoculation and co-cultivation with *Agrobacterium* has been shown to improve genetic transformation frequencies in some woody fruit plants, such as plum (Mante et al., 1991) and apricot (Machado et al., 1992). Sangwan et al. (1992) have also reported that the number of putatively competent cells for transformation was greatly increased by a pre-culture treatment in *Arabidopsis thaliana* explants on an auxin-rich medium. In this study, a three-day co-cultivation period was found to be optimum for enhanced wheat transformation. More than 3 days co-cultivation resulted in abundant proliferation of the bacteria and a subsequent decrease in the regeneration frequency of transformed shoots. However, in some other crops co-cultivation periods of more than three days have been successfully used (Dong et al., 1991; Mourgues et al., 1996).

CONCLUSION

The optimum protocol for transformation and regeneration of the two wheat cultivars was: callus induction on MS medium supplemented with 4.0 mg/l 2,4-D, pre-culturing in MS medium containing 4.0 mg/l 2,4-D and 1.0 mg/l Zeatin for eight days, Inoculation with *Agrobacterium* suspension (OD_{600} 0.1), co-cultivation in PGRs-free MS medium containing 300 mg/l Cefotaxime in combination with 50 mg/l Vancomycin for three days. Pre-selection and shoot initiation was achieved in PGRs-free MS medium, while selection was carried in MS medium containing 4.0 mg/l 2,4-D, 1.0 mg/l Zeatin, 300 mg/l Cefotaxime, 100 mg/l Vancomycin and 15mg/l (Elnielain) or 25 mg/l (Bohain) Hygromycin for four-six weeks. The putative transformed shoots were elongated in MS medium containing 4.0 mg/l 2,4-D then transferred to a MS medium with 1.0 mg/l 2,4-D, 1.0 mg/l GA_3 and1.0 mg/l Zeatin for root induction. This protocol resulted in

transformation efficiency of 20% for cv. Bohain and 23% for cv. Elnielain. The developed protocol may be used to introduce genes for heat or drought tolerance or genes for resistance to a particular biotic stress in order to stabilize yield and improve productivity under Sudan's conditions.

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