Transgenic Plant Journal ©2011 Global Science Books

Progress and Prospects for *Agrobacterium*-Mediated Genetic Transformation in Sorghum in Comparison to Other Cereals

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ABSTRACT

Transgenic technology in sorghum, *Sorghum bicolor* L. (Moench), especially the *Agrobacterium*-mediated method, has picked up momentum in the recent past. High throughput *Agrobacterium*-mediated transformation in sorghum is prerequisite for use as a tool in functional genomics through T-DNA insertional mutagenesis and for development of marketable transgenic crop products. Despite equivalent efficiency in transient expression with other cereals, recovery of transgenic plants in sorghum is quite low, which can be attributed to a loss of regeneration potential over subsequent prolonged subculture and treatment with agents for decontamination of *Agrobacterium*. It can be overcome by right-choice of starting material with high regeneration and effective *in vitro* culture methods. Immature inflorescences are resourceful for high regeneration in sorghum, but recovery of plants subsequent to *Agrobacterium*-based methodology is very low. *In vitro* methods based on seed as the starting material in sorghum stand inefficient. Alternatively, immature embryos are frequently used explants for genetic transformation and these protocols need further improvement for increasing the efficiency of transformation in sorghum. Thus, the critical factors in *Agrobacterium*-mediated genetic transformation in sorghum include i) moderation of *Agrobacterium*-infection parameters ii) decontamination procedures with least phytotoxic effects iii) efficient plant regeneration and iv) specialized vectors for high efficiency transformation. The current review presents the achievements in *Agrobacterium*–based genetic transformation of sorghum and possible recommendations in the light of developments in other cereals.

Keywords: Acetosyringone, anti-microbial agents, host strains, *in planta*, surfactants

CONTENTS

INTRODUCTION

Sorghum is the fifth important cereal in the world and is used as food, fodder, fuel and feed. Sorghum crop is bestowed with a great diversity within and can serve as a source for mining useful genes for crop improvement programs. It is an attractive system for functional genomics because of its minimum levels of gene duplication and would serve as a repository for not only the grasses, but also for improvement of crop species worldwide. Transgenic technology in sorghum, especially mediated through *Agrobacterium*, has picked up momentum in the recent past. Efficient *Agrobacterium*-mediated genetic transformation serves as a tool for development of transgenic crop varieties and also for analysis of gene functions through insertional mutagenesis using T-DNA. Today many important plant species are routinely transformed using *Agrobacterium*. The interest in using this organism towards plant genetic transformation of crop species is expanding because of its complete gene integration and low input costs. A number of reviews on *Agrobacterium*-mediated transformation in cereals (see reviews by Wei *et al.* 2000; Shrawat and Lorz 2006), and with special emphasis on rice (Roy *et al.* 2000), wheat (Cheng *et al*. 2004), were published, but have not emphasized on sorghum. In this review, *Agrobacterium* mediated genetic transformation in sorghum is dealt in detail. Decontamination in sorghum is a serious hurdle and methods of decontamination employed in other crops are accounted elaborately so as to reach an optimum procedure for early and complete removal of *Agrobacterium* to obtain plant regeneration. Other contributing factors such as pre-incubation and media constituents during co-cultivation were discussed.

IN VITRO CULTURE IN SORGHUM

Efficiency of *Agrobacterium* method is dependent on tissue culture response of crop species. Sorghum is considered recalcitrant among cereals for *in vitro* culture, thereby genetic transformation (Subudhi and Nguyen 2000). Recalcitrance

Received: 23 December, 2009. Accepted: 29 September, 2010.

Review

Table 1 Details of genetic transformation in sorghum.

Gene (s) introduced	Method of transformation	Explant	Genotype	Efficiency of transformation %	Reference
Gus and nptII	Protoplast electroporation	-	NK300	-	Battraw and Hall 1991
Gus, *hph*, *nptII*	Bombardment	Immature embryos*	-	-	Hagio *et al.* 1991
bar and *gus*	Bombardment	Immature embryos	P898012	0.08	Casas *et al.* 1993
bar and *gus*	Bombardment	Inflorescences	P898012	0.33	Casas *et al.* 1997
bar and *chiII*	Bombardment	Immature embryos	Tx430, SRN 39	0.09	Zhu *et al.* 1998
bar and *gus*	*Agrobacterium*	Immature embryos	P898012, PHI 391	2.1	Zhao *et al.* 2000
bar and *gfp*	Bombardment	Immature embryos	P898012	1.0	Able *et al.* 2001
bar and *chiII*	Bombardment	Immature embryos	Tx430, SRN 39	-	Krishnaveni *et al.* 2001
bar and *gus*	Bombardment	Immature embryos	Tx430	0.18	Emani *et al.* 2002
bar and *gus*	Bombardment	Immature embryos	214856, 213108	1.3	Tadesse *et al.* 2003
Gfp, *G11*, *tlp*	*Agrobacterium*	Immature embryos	Tx430, C401 and wetland	-	Jeoung *et al.* 2002
Gfp and *tlp*	*Agrobacterium*	Immature embryos	Tx430, C401	2.5	Gao *et al.* 2005a
*man*A	*Agrobacterium*	Immature embryos	P8505, C401	2.88, 3.3	Gao *et al.* 2005b
Bt cry 1Ac	Bombardment	Shoot apices	BTx623	1.5	Girijashankar *et al.* 2005
nptII and gusplus	*Agrobacterium*	Immature embryos	Tx430, C2-97	0.3 to 4.5	Howe *et al.* 2006
Gus and *hpt*	*Agrobacterium*	Immature embryos	Sensako 85/1191	5.0	Nguyen *et al.* 2007
Sgfp and *man*A	*Agrobacterium*	Immature embryos	P898012	7.7	Gurel *et al*. 2009
Bar and *Bt cry1 Aa* and *cry1B*	Bombardment	Immature embryos	CS3541 and 296B	0.1-0.3	Visarada *et al.* 2009

* Reported from a suspension culture of *Sorghum vulgare*

bar: phosphinothricin acetyl transferase, *chiII*: Rice chitinase, *gfp*: green fluorescence protein, *gus*: β-glucuronidase, *hph*: hygromycin phosphotransferase, *manA*: phosphomannose isomerase, *nptII*: neomycin phosphotransferase, *tlp*: thaumatin-like protein

in sorghum can be attributed to i) release of polyphenols into the culture medium ii) low regeneration iii) rapid decline of regeneration subsequent to subculture after transformation and iv) lack of model genotypes in sorghum for genetic transformation that ease the adoption of the transgenic technology to a crop species. Immature inflorescences and immature embryos provide actively dividing cells that are readily accessible for genetic transformation. Immature inflorescences of sorghum are practical source of young tissues and are best suited explant in terms of callus induction as well as plant regeneration. These are easy to isolate and culture *in vitro*. They can withstand physical damage during handling, unlike immature embryos, which fail to respond in damage. Amoah *et al*. (2001) reported an additional advantage of less variation of plants after regeneration. However, in our experiments, plants could not be regenerated after transformation using the above explants. We observed that despite their initial high regeneration potential, lack of sustainability of regeneration for longer periods and loss of regeneration subsequent to selection, are serious impedements for application of immature inflorescences as the target tissues in transgenic technology.

To date, recovery of transgenic plants through *Agrobacterium*-method and particle bombardment has been achieved chiefly with the aid of immature embryos in sorghum (**Table 1**). Immature embryos to date occupy the most-favored explant sources for *Agrobacterium* based system in other cereals as well (**Table 2**). Zhao *et al.* (2000) reported that formation and maintenance of good quality callus from immature embryos as one of the most critical factors for efficient transformation. Among the explants of sorghum, the percentage of transient *gus* expression after *Agrobacterium* infection, was reported highest in immature embryos (Visarada *et al.* 2003). Carvalho *et al.* (2004) studied tissue culture response of immature embryos during and after co-cultivation and identified four critical factors: (i) sensitivity of immature sorghum embryos to *Agrobacterium* infection, (ii) growth conditions of donor plant, (iii) type of explants and (iv) co-cultivation medium. Cold treatment (one day at 4°C) of immature seeds before isolation of immature embryos has increased the callus induction (Nguyen *et al.* 2007). Lu *et al.* (2009) obtained highest transformation efficiency with green house grown embryos, whereas Zhao *et al.* (2000) reported field grown embryos were significantly higher than those using greenhouse-grown embryos. The ideal size and age of the embryos were found to be 1.0-1.5 mm or 9-14 d after pollination (Zhao *et al*. 2000; Nguyen *et al.* 2007; Gurel *et al.* 2009; Lu *et al.* 2009). Immature embryos are available for a short period in a year during the crop season, isolation and culture of immature embryos in sorghum, rice and wheat is a long drawn tedious process. Manual isolation of immature embryos under laboratory condition followed by sterilization under clean air bench with the help of metallic filters allows rapid processing without causing chemical and physical damage to the embryos and results in 95-100% germination (Raju *et al.* 2009). Thousands of immature embryos in sorghum can be isolated, pre-cultured and deployed within the short period of crop season for conducting large-scale genetic transformation. Protocols based on mature seeds are most convenient for *in vitro* research, as seeds can be stored, easy to handle and are available round the year. However, poor *in vitro* response and frequent contamination in sorghum (as the seed is naked) and susceptibility to infestation of many fungi during the seed maturation stages make it a less dependable choice for genetic transformation experiments. Shoot apices and shoot apical meristems isolated from 3-7 d old germinated seedlings stand as alternative. Though regeneration of calli derived from shoot tips was shown promising, genetic transformation especially the *Agrobacterium* based methods are not reported so far, the reasons being lack of sustainability in regeneration. However, isolation of shoot apical meristems and induction of multiple shoots is followed by many research groups in sorghum (Zhong *et al.* 1998; Harshavardhan *et al.* 2002; Syamala and Devi 2003; Maheswari *et al.* 2006; Saikishore *et al.* 2006b). Shoot meristem-based systems are; rapid, minimize the variation due to somaclonal variation, genotype independent and thus can be extended to choice genotypes. Isolation of meristems in monocots requires careful dissection and removal of leaf primordia. These meristems were found promising in genetic transformation of rice (Park *et al.* 1996; Arockiasamy and Ignacimuthu 2007) and maize (Sairam *et al.* 2004). Method for rapid isolation of shoot apical meristems and culture in sorghum are described by Saikishore *et al.* (2006b).

Genotype specific response to genetic transformation and *in vitro* culture exist in many plant species. Responsive genotypes serve as start material for introgression of the transgene. Lack of such model genotypes in sorghum is one of the factors for slow progress of genetic transformation research (Saikishore *et al.* 2006a). Public line of sorghum, P898012 and Tx430 were most often reported sorghum genotypes (**Table 1**). Release of phenolic compounds into the culture medium is frequent in sorghum, which is detri-

Table 2 Salient points from recent *Agrobacterium*-mediated protocols of other cereals.

Crop	Transformation efficiency (host strain)	Explants	Points to ponder	References
Rice				
Group 1 recalcitrant indica rice	738% (LBA4404)	Immature embryos	Efficiency of gene transfer varied with the kinds of gelling agents and the basic compositions of co-cultivation media. Sectioned the embryo into as many as 30 pieces after non-selective cultures following co-cultivation.	Hiei and Komari 2006
Japonica cultivars Indica cultivar, Kasalath	-	Immature embryos Mature seed	Immature embryos were pretreated with heat (43°C) and centrifugal force (20,000 g for 10 min), and then infected with *Agrobacterium.*	Hiei and Komari 2008
Japonica varieties	30-50%	Mature seed	Transgenic plants obtained in 2-3 months Carbenicillin and Claforan (250-500 mg/L) used for decontamination. Blotting the calli after infection for complete removal of excess *Agrobacterium*-important to remove excess water from calli to ensure active plant culture growth. Co-cultivation and incubation at 28°C in the dark for 48–60 h.	Nishimura *et al.* 2006
IR-64	9.2 (LBA4404) 12.1 (EHA105)	Embryogenic callus	Anti necrotic compounds (L-Cysteine, Ascorbic acid) were used in co-cultivation along with phenolic compound, the acetosyringone. Exogenous addition of spermidine (0.1 M) improved regeneration.	Saha *et al.* 2006
PB-1 Co-43 White ponni ADT 38	0.9–5.2% (LBA4404)	Embryogenic callus derived from mature seed	Calli blotted, dried and co-cultivated. Cefotaxime (250 mg/L) was used for decontaminating *Agrobacterium.*	Nanda Kumar *et al*. 2007
White Ponni PB-1	5.6-6.2% (EHA101)	Shoot apex	Four day old shoot apices were used for transformation. Co-cultivation was carried out at 25°C, 3 days at pH 5.8. Cefotaxime (250 mg/L) with drop of Tween 20 was used for decontamination.	Arockiasamy and Ignacimuthu 2007
Wheat				
Ofanto (*T. durum*)	9.7% (AGL-1)	Immature embryos	Additional *vir* genes (*vir* G alone and combination of *vir*B, *vir*C and *vir*G) contributed for high transformation efficiency. Timentin (150 mg/L) was used in controlling growth of *Agrobacterium.*	Wu *et al*. 2008
PDW215 (*T. durum*)	3% (LBA4404)	Mature embryos	Use of acetosringone during inoculation. Cefotaxime (250 mg/L) for decontamination.	Vishnudasan *et al*. 2005
HD2329, CPAN1676, PBW343 (*T. aestivum*) PDW215, WH896 (*T. durum*)	1.28 to 1.7% (LBA4404)	Mature embryos	Acetosyringone and glucose were employed in both inoculation and co-culture/co-cultivation medium.	Patnaik *et al.* 2006
Maize				
Inbred lines B104, B114 KY21	2.8-8%	Immature embryos	*Agrobacterium* suspension precultured in Infection +AS medium (2 h), was diluted to OD_{550} 0.3–0.4 and added (1 ml) to the dissected embryos that were prewashed in bacteria-free Infection +AS medium. All co-cultivation media were supplemented with 300 mg/L cysteine, Carbenicillin (250 mg/L) was used to counter select bacteria in resting and selection media. MS medium promoted post infection growth of transformed tissues. Media that enhanced post-infection embryogenic callus induction frequency and promoted vigorous callus growth on responding embryos was recommended	Frame *et al*. 2006
LPC13	6.8% (EHA105)	Immature embryos	Physical microwounds (bombardment, sonication) before and during *Agro* infection promoted T-DNA transfer. High Embryogenic calli were generated by the addition of Silver nitrate in N_6 based induction medium.	Oritz *et al*. 2007
A188, A634, H99 and W117	50% 15%	Immature embryos A1888 A634, H99, W117	Use of immature embryos at correct developmental stage (8-15 days after pollination, 1.0-1.2 mm in length). Collect immature embryos from vigorous plants. 3 months from infection to transplantation. Pretreatment with heat (46°C for 3 min in water bath) and centrifugation	Ishida *et al.* 2007
Hi-II	12-18%	Immature embryos	Low salt media with L-Cysteine or L-Cysteine with DTT during the infection stage.	Vega *et al*. 2008

mental to regeneration. Frequent subculture of calli, addition of activated charcoal, PVP (polyvinyl pyrrolidone), PVPP (polyvinyl poly pyrrolidone) and coconut water to the culture medium were reported to decrease the pigment production and mitigate the influence of phenolics. Culture of immature embryos on N_6 medium not only decreased callus response (76% with MS and 20% with N_6) but also increased the production of phenolic pigments in sorghum (Zhao *et al.* 2000; Saikishore *et al.* 2006b). Gao *et al.* (2005b) reported an enhanced pigment production in embryo derived calli after *Agrobacterium* infection with overgrowth of bacterium on the explants. Addition of coconut water, proline and asparagine to the culture medium were found to reduce the pigment production, and increase the callus growth and percentage (Zhao *et al.* 2000; Carvalho *et al.* 2004). Addition of 1% (w/v) PVP throughout the culture period mitigated tissue blackening in sorghum benefited transformation frequency as necrosis and browning during

the culturing process is detrimental to both regeneration and transformation (Gurel *et al.* 2009; Lu *et al*. 2009). Recently, Lu *et al.* (2009) noticed high levels of phosphinothricin (5-10 mg/L) accelerated production of phenolic compounds and embryo browning that prevented recovery of transgenic sorghum. And that PVPP was superior to PVP in preventing browning. We corroborate the view of Ishida *et al.* (2007) that an experience in tissue culture is necessary to establish and maintain genetic transformation of cereals.

GENETIC TRANSFORMATION OF SORGHUM

Though tissue culture response of sorghum was reported long time ago, corresponding developments in terms of genetic transformation lagged behind because transformation followed by regeneration is extremely tricky in sorghum. Nevertheless, it has picked up momentum in the past few years (Girijashankar and Swathisree 2009). All the 3 types of genetic transformation methods *viz.,* (i) protoplast mediated transformation (Battraw and Hall 1991) (ii) particle bombardment (Hagio *et al*. 1991; Casas *et al*. 1993, 1997; Girijashankar *et al*. 2005) and (iii) *Agrobacterium*-mediated method (Zhao *et al*. 2000; Gao *et al*. 2005a, 2005b; Howe *et al.* 2006; Nguyen *et al*. 2007; Gurel *et al.* 2009; Lu *et al.* 2009) were reported in sorghum. To date, reports on genetic transformation of sorghum through particle bombardment outnumbered the reports through *Agrobacterium* infection (**Table 1**). Salient points from high efficiency *Agrobacterium*-mediated genetic transformation in other cereals such as rice, wheat and maize are provided in **Table 2**. Parameters used in *Agrobacterium*-mediated genetic transformation of sorghum are described in **Table 3**.

AGROBACTERIUM-MEDIATED PROTOCOLS

Godwin and Chikwamba (1994) first reported inoculation of sorghum meristem tissue with *Agrobacterium.* Success of *Agrobacterium* mediated transformation at a much later date by Zhao *et al.* (2000) has added new direction in the application of using this organism in sorghum. Recently Gao *et al*. (2005a, 2005b), Howe *et al.* (2006), Nguyen *et al.* (2007), Gurel *et al.* (2009) and Lu *et al.* (2009) reported successful recovery of transgenic sorghum plants through *Agrobacterium*. Zhao *et al.* (2000) recommended 4 preliminary factors for initial standardization of transformation as media, antibiotics, T-DNA delivery and effect of *Agrobacterium*-infection on callus response. Their results provided baseline conditions in subsequent experiments to achieve stable transformation of sorghum using *Agrobacterium* with overall transformation frequency of 2.1%. Nguyen *et al.* (2007) reported an increase in efficiency to 5% by addition of activated charcoal in the callus induction medium, pretreatment of immature seeds, callus induction in ventilated Petri dishes and regeneration in plastic boxes (instead of Petri dishes to increase ventilation). Gurel *et al.* (2009) could achieve as high transformation efficiency as 8.3% through pre-heat shock (43°C, 3 min) and cooling (25°C) of immature embryos prior to infection. Lu *et al*. (2009) recovered transgenic shoots of sorghum by the use of phosphinothricin at a low concentration (2.5 mg/L) with decreased duration of selection for 4-8 weeks. In maize additional factors like *Agrobacterium* concentration, duration of co-cultivation and resting period were identified and balancing these factors against one another was reported critical for high throughput transformation system (Zhao *et al.* 2001). Efficient (15-50%) *Agrobacterium*-transformation in maize was reported to be the result of selection of healthy immature embryos from vigorous plants (Ishida *et al.* 2007). Jones *et al.* (2005) reviewed the current methods of *Agrobacterium* transformation in wheat and described a detailed account of a robust protocol. The authors opined that automation of transfer steps coupled with manipulations of bacterium suspensions would enable a higher throughput that would allow production of significantly more transgenic lines.

Preparation of *Agrobacterium* culture for infection

A ready-to-use *Agrobacterium* inoculum should supply robust and physiologically active bacterial cells for efficient T DNA transfer. Density of the culture inoculum should facilitate optimum transformation efficiency and minimum tissue damage. Zhao *et al.* (2000) used 1×10^9 cfu/ml (colony forming units) (OD_{550} = 0.70) and 0.5×10^9 cfu/ml (LBA4404) for inoculation in sorghum. Gao *et al.* (2005a) recommended a low density of culture (OD_{600} = 0.2 (EHA105)) in sorghum during inoculation followed by culture at 25°C for 3 weeks. Similarly, Nguyen *et al.* (2007) used low density cultures of OD_{600} = 0.1-0.3 (LBA4404) for transformation experiments with sorghum immature embryos. Gurel *et al.* (2009) (EHA101 and LBA4404) and Lu *et al.* (2009) used OD_{550}= 0.4 for infecting the sorghum tissues and for generating transgenic plants in lines of P898012. We tested 4 different densities (OD_{600}) of the culture for infection *viz*. 0.5, 1.0, 1.5 and 2.0. Though there were significant differences in transient *gus* expression between 0.5 and 1.0, the latter producing higher transformation, differences were not significant beyond 1.0 OD_{600}. Since the transient expression efficiency is not directly proportional to the recovery of transgenic plants, it is helpful to use low density culture (OD_{600} = 0.5) (Kavitha 2005). In similar lines, Gao *et al.* (2005a) reported an increase in transformation frequency in sorghum with increase in bacterial concentration, but observed increase in tissue damage with high bacterial densities. Though transient expression increases, in general, with increase in cell density necrosis of the infected tissues due to overgrowth of bacterium is a common experience reported by many. Balancing the above two parameters is important in recovery of plants after *Agrobacterium* mediated transformation. Preconditioning of explants *in vitro* such as osmotic treatment with increased concentration of sucrose and addition of glucose is reported in rice and maize (Hiei *et al.* 1994; Zhao *et al.* 2001; Opabode 2006). Such osmotic pretreatment can be explored in sorghum for increasing the transformation efficiency. Pretreatment of immature embryos with heat shock (43°C, 3 min) followed by cooling (25°C) helped in achieving high transformation efficiency in sorghum (Gurel *et al.* 2009).

Addition of surfactants like Pluronic F68 (0.03%) to the inoculation medium was found useful in sorghum (Carvalho *et al.* 2004). Similarly, addition of Silwet L-77 (0.04%) and Pluronic acid-F68 (0.02%) during co-cultivation was reported effective in wheat (Cheng *et al.* 1997; Wu *et al.* 2003). Co-cultivation with L-cysteine (400 mg/L) lead to an improvement in transient *gus* expression in sorghum and stable expression in maize (Frame *et al.* 2002; Saikishore *et al.* 2004; Vega *et al.* 2008). Presence of L-cysteine promoted the plant growth and greater recovery of plants in our study. Contrastingly, addition of antioxidants L-cysteine and Dithio threitol (DTT) caused significantly reduced transformation frequency in sorghum (Lu *et al.* 2009). Addition of proline, culturing and regeneration at 32-33°C for (except during co-cultivation) contributed for high-speed *Agrobacterium* transformation in rice in which transgenic plants were regenerated within a month by infection in early stages of culture of scutellum-derived calli (Toki *et al.* 2006). In barley, addition of $CaCl_2$ (< 3 mM) enhanced the transformation efficiency (Kumlehn *et al.* 2006). Addition of coconut water to the co-cultivation promoted fast embryo response and increased the embryo survival in sorghum (Zhao *et al.* 2000). Embryos were immersed in the bacterial inoculation medium for 5-10 min, blotted with a filter-paper to remove excess bacteria and co-cultivated on acetosyringone containing coconut water to obtain high transformation efficiency (Carvalho *et al.* 2004). In sorghum, acetosyringone is often used in the range of 100-200 µM (**Table 3**). Jeoung *et al.* (2002) tested different concentrations (200-1000 µM) of acetosyringone on the efficiency of *gfp* expression in sorghum and reported an increase in expression with increase in the concentration of acetosyringone. Optimum concentration of acetosyringone and duration of infection

Table 3 Parameters used in *Agrobacterium*-mediated transformation of sorghum.

Agrobacterium strain	Conc. of acetosyrigone (µM)	Vector type	Infection time/co-cultivation time, temperature	Plant selection agent	Concentration of selection agent (mg/L)	Reference
LBA4404	100	pSB 1 and pSB 11	5 min/3 or 7 d	PPT	1.5-10	Zhao *et al.* 2000
AGL-1, EHA101, EHA105	50-1000	pZP200, pCAMBIA 1300	3 d	Bialaphos	2-3	Jeoung *et al.* 2002
LBA4404	200	pTOK 233	2 or 5 d, 25°C	Hygromycin	50	Carvalho *et al.* 2004
EHA101	100	pPZP201	10 min/3 d	*gfp*	NA	Gao *et al.* 2005a
EHA101	100	pPZP201	-	Mannose	1-3%	Gao *et al.* 2005b
C58 NTL4/Chry5	200	pPTN290	5 min/ 2 d	Kanamycin	10, 20	Howe *et al.* 2006
LBA 4404	100	pCAMBIA 1301	5 d	Hygromycin	5	Nguyen *et al.* 2007
EHA101 and LBA4404	100	pPZP201-GFP-PMI (pGFP-PMI)	15 min	Mannose	1-3%	Gurel *et al*. 2009
EHA105	200	pCAMBIA 3300	10 min/3 d, 26°C	PPT	3-5	Visarada *et al.* 2009

NA: Not applicable, *Gfp*: Green fluorescent protein, PPT: Phosphinothricin

(5-30 min) varied with the *Agrobacterium* strain and sorghum inbred combination.

Use of anti-microbial agents for decontamination of *Agrobacterium*

Residual *Agrobacterium* cells from the infected tissues are removed by decontamination through washes and supplementation of antibiotic in the medium after co-cultivation. Overgrowth of *Agrobacterium* on explant tissues reduces the supply of oxygen and respiration of the tissues is prevented. Thus, the calli turn necrotic and loose the potential to regenerate to whole plants. Elimination of *Agrobacterium* from cultures during the early stages of selection is an important factor. As a measure to control bacterium, the explant tissues are washed or treated with several antibiotics. Some of the measures employed for permanent and rapid decontamination of *Agrobacterium* are (i) low density of the culture used for initial infection (ii) frequent antibiotic washes and (iii) addition of antibacterial agents in selection and regeneration media.

Several antibiotics such as cefotaxime, carbenicllin, timentin and ticarcillin are used following co-cultivation either alone or in combination for effective removal of *Agrobacterium.* Right choice and appropriate combination of antibiotics is critical not only for decontamination, but also for subsequent regeneration. Details on the mode of action of antibiotics help in deciding the right prescription for decontamination (**Table 4**). Ogawa and Mii (2004) examined the *in vitro* anti bactericidal concentration of β-lactams against *Agrobacterium* host strains, LBA 4404 and EHA 101, host strains of *Agrobacterium.* They found that cefotaxime, cefbuperazone and meropenem are active against LBA4404 while meropenem alone is active against EHA101. Growth of AGL-1 strain was effectively suppressed by the use of moxalactum, a β-lactam antibiotic that also worked as a promoter agent for somatic embryo formation in cacao (Mayolo *et al.* 2003). Effects of these decontaminating adjuvants on *in vitro* culture of sorghum are not studied in detail. Direct comparison of these effects is difficult because they vary with the explant tissues, culture system and plant species.

In successful recovery of transgenic sorghum plants, Zhao *et al.* (2000), Gao *et al.* (2005a, 2005b), Nguyen *et al.* (2007) Gurel *et al.* (2009) and Lu *et al.* (2009) used 100 mg/L carbenicillin in their experiments and could effectively decontaminate the tissues. Yao *et al.* (2004) found that cefotaxime was more inhibitory to callus induction in sorghum as compared to carbenicillin and ampicillin. According to Carvalho *et al.* (2004) combination of coconut water in the co-cultivation medium, encouraged vigorous, actively growing immature embryos and the removal of excess bacteria, both factors are both critical for success of transgenic technology. Ieamkhang and Chatchawankamph (2005) recommended fresh solution of 300 mg/L augmentin and timentin to be used within 3 weeks of preparation in tomato transformation. Use of ampicillin as a bacterial selection marker in *Agrobacterium* transformation vectors compromises the preferred treatment for *Agrobacterium* elimination (Barrell and Conner 2006). Apart from antibiotics other antimicrobial agents are added to the culture media after co-cultivation for arresting the growth of the bacterium (**Table 4**). Kavitha (2005) compared 12 non-antibiotic treatments in 4 host strains, *viz*., AGL-1, C58, EHA 101 and EHA 105 of *Agrobacterium* for control of *Agrobacterium* overgrowth. A strain specific response to treatment was observed. Media adjuvants in the order of silver nitrate > cuper sulphate > lysozyme > PVPP (50 mg/L) could effectively control in all the 4 strain, while weak acidic culture conditions (pH) were ineffective to control *Agrobacterium*.

Efficiency of *Agrobacterium* transformation

Efficiency of transformation reported so far ranged from 0.08 to 7.7% in sorghum (**Table 1**). Zhao *et al.* (2000) first showed 2.1% transformation efficiency produced 131 stably transformed events from 6175 immature embryos transformed through *Agrobacterium*. They reported that field grown embryos had higher transformation frequency than greenhouse-grown sorghum embryos. Gao *et al.* (2005a) obtained a total of 1011 fertile transgenic plants from 61 independent callus lines using *gfp* as visual screening marker. For the first time the authors demonstrated the use of *gfp* gene and GFP screening for efficient production of stably transformed sorghum plants without use of antibiotics or herbicides as selection agents with a transformation frequency of 2.5%. Similarly, Gao *et al.* (2005b) could recover as many as 167 transgenic plants from 9 different embryogenic callus lines. Use of *gfp* as visual selection marker allowed visual selection of transformed tissues from early steps along with *pmi* (phosphomannose isomerase) as plant selection marker. The authors showed an increase in transformation efficiency of 2.88% in the genotype P8505 and 3.3% for C401. Necrosis and damage that caused the low regeneration in each by *Agrobacterium* overgrowth amounted to 49.2% in P8505 and 18% in C401. The authors recommended the use of 1-2% mannose to reduce necrosis during selection. Among the T_0 plants 33.3% had single copy and 66.7% had two or more copy insertions. Howe *et al.* (2006) devised a rapid *Agrobacterium*-mediated transformation protocol using *Agrobacterium* strain, C58 with novel chromosomal background, NTL_4/Chry5 and the transformation frequencies ranged from 0.3 to 4.5% with an average frequency of 1% for tested genotypes in sorghum immature embryos. Nguyen *et al.* (2007) could increase the transformation efficiency in sorghum to 5% by improving the regeneration system. Recently, Gurel *et al.* (2009) attributed their high transformation efficiency (up to 8.3%) in sorghum through pretreatment of target tissues with heat shock and cooling that lead to changing fluidity of the membrane, which impacted the susceptibility to infection.

Table 4 Anti-microbial agents used for decontamination of *Agrobacterium.*

Anti-microbial agent	Category	Mode of action	Concentration	Remarks*
Carbenicillin	β-lactam antibiotic, a penicillin derivative.	Inhibits cross linking of peptidoglycan and prevents bacterial cell wall synthesis	100 mg/L	• Non-toxic to plant cells • Less sensitive to low pH. • Sensitive to β-lactamases. • Should be used in combination with β-lactam resistant compounds.
Cefotaxime	Semi synthetic analog of Cephalosporin, third generation antibiotic	Disrupts synthesis of peptidoglycan and prevents bacterial cell wall synthesis	250 mg/L	• Sensitive to β-lactamases. • Should be used in combination with β-lactam resistant compounds
Claforan	Trade name of Cefotaxime sodium	Broad spectrum activity	400 mg/L	• Highly stable in presence of β-lactamases.
Timenitin (or) Ticarcillin	β -lactam antibiotic, is a carboxy penicillin used in combination with clavulante as timentin	Prevents cross linking of peptidoglycan during cell wall synthesis	300 mg/L	• Stable in light Ling *et al*. (1998) • Sensitive to β-lactamases. • Used in combination with clavulanic acid.
Vancomycin	Tricyclic glycosylated non ribosomal peptide (Glycopepetide)	Acts by inhibiting the formation of peptidoglycan polymers of the bacterial cell wall.	250 mg/L	• Used in combination with carbenicillin and cefotaxime.
Clavulanic acid	β-lactam antibiotic, a penicillin derivative	β-lactamase inhibitor		• Used in combination with Ticarcillin.
Meropenem	β-lactam antibiotic, a penicillin derivative	Bactericidal. Inhibits bacterial cell wall synthesis like other β -lactams.	0.5 mg/L	• Effective suppressive agent of *Agrobacterium* found in Orchid transformation
Amoxycillin	β-lactam antibiotic, a penicillin derivative	Inhibits cross linking of peptidoglycan and effects the bacterial cell walls	100-300 mg/L	• Susceptible to β-lactamase. Used in combination with clavulinic acid.
Moxalactum	β-lactam antibiotic Cephalosporin	Usually grouped with cephalosporins	200 mg/L	• Promoted somatic embryogenesis in cacao.
Augmentin	Combination of antibiotics containing amoxicillin and clavulanic acid in the form of potassium clavulante	Increased spectrum of action.	300 mg/L	• Solution prepared prior to use. • No toxicity found in tomato.
Non antibiotic agents=				
Anti-microbial agent	**Category**	**Mode of action**	**Concentration**	**Remarks***
Copper sulphate	Inorganic salt	Not known	Inorganic salt	Lower levels (20-50 mg/L) of copper sulphate in culture medium induce plant regeneration. (Kavitha 2005)
Silver nitrate	Inorganic salt	Not known	5 -30 mg/L	• Sensitive to light. • Enhances the induction of direct somatic embryogenesis • Promotes *in vitro* regeneration due to anti ethylene activity. • Stimulates direct shoot regeneration. • Inhibits *Agrobacterium* growth after co-cultivation and during regeneration. (Kavitha 2005, Oralikowska 1997)
Lysozyme	Enzyme	Damages bacterial cell walls by catalyzing hydrolysis of 1,4- β –linkages.	2 mg/L	Lysozyme at pH 7.0 is a good decontamination agent without adverse effects on tissue culture (Kavitha 2005; Negri and Manzechi 2002).
PVPP	Cross linked polyvinyl pyrrolidone	Not known	1%	An antioxidant, protects the cell cultures (Kavitha 2005)
Cysteine	Amino acid	Not known	40 and 200 mg/L	• Antioxidant • Increases cell viability (Enríquez-Obregon *et al.* 1999)
Ascorbic acid	Vitamin C	Not known	20 mg/L	• Antioxidant • Increases cell viability (Enríquez-Obregon *et al.* 1999)
pH 4.5, and 5.5	-	Not known	-	Low pH washes containing cefotaxime in the substrate were the most effective, but had inhibitory effects on plant cell proliferation. (Kavitha 2005; Negri and Manzechi 2002)

* Relevant references are cited in the text.

In planta transformation

In planta method of genetic transformation using *Agrobacterium* obviates the need for plant regeneration *in vitro*. These methods are developed chiefly in dicots, while in monocots they are in preliminary stage. Floral dip in sorghum with a low density culture (<0.1 OD_{600}) before anthesis was advantageous for better seed set, but resulted in localized expression of *gus* in endosperm tissues and not in scutellum (Kavitha 2005). Elkonin *et al*. (2005) devised a simple and effective method for obtaining sorghum plants by *Agrobacterium* mediated transformation *in planta* by dropping *Agrobacterium* cell suspension on the surface of the stigma of pre-fertilized florets. Out of 667 seedlings selected on kanamycin 4 plants have survived and 3 expressed *gus* activity in the shoot tillers. Though the transformation

Table 5 Transformation of agronomically important traits in sorghum.

Transgenic trait	Transgene (s)	Method	Explant	Reference
Resistance to stem-borer	*Bt cry1A (c)*	Bombardment	Shoot tip	Girijashankar *et al.* 2005
Resistance to stalk root	*Rice chitinase*	*Agrobacterium*	Immature embryos	Zhu *et al.* 1998
Resistance to stalk root	*chi 11*	Bombardment	Immature embryos	Krishnaveni *et al.* 2001
Enrichment of lysine	*dhdps-r1*	Bombardment	Immature embryos	Tadesse *et al.* 2004
Resistance to stem-borer	*Bt cry1Aa and cry1B*	Bombardment	Immature embryos	Visarada *et al.* 2009

dhdps-r1: Dihydro-picolinate synthase; *chi11*: Rice chitinase

frequency is low, the advantage of this type of transformation *via* pollen tube pathway excludes chimerity of transgenic plants that is possible using multi cellular explants. Development of methods based on *in planta* is desirable in low investment crops like sorghum because of a very low regeneration post transformation and selection.

FUTURE PROSPECTS

Development of GM sorghum to impart resistance to biotic and abiotic stresses and for value addition helps in realizing high productivity with further low inputs. Details of agronomically important traits introduced in sorghum through genetic transformation are listed in **Table 5**. A routine and efficient (1%) transformation for crop improvement in sorghum, equivalent to the reports of rice and maize, is possible through key factors: i) protocols for *Agrobacterium*-infection with optimum efficiency and without tissue damage; ii) employing decontamination procedures with least phytotoxic effects; iii) efficient plant regeneration system that allows recovery of plants after passing through the cycles of selection and; iv) design of specialized vectors to render high efficiency transformation. With the advent of improved *Agrobacterium*-mediated transformation and regeneration technology it will be possible to create large insertional line populations in sorghum in near future as of other crops like rice and maize (Balyan *et al.* 2008).Generation of high-throughput technology can benefit in lowering the cost of sorghum and ca benefit the crop improvement programs worldwide through contribution of many useful genes.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support through research grants from Indian Council of Agriculture Research (NPTC 3048) and Andhra Pradesh-Netherlands Biotechnology program. We are thankful to Ms A. Annapurna for her help in preparation of the MS.

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