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Impact of cefotaxime on *Agrobacterium* mediated BT gene transformation in sugarcane

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Abstract
Complete elimination of *Agrobacterium* from plant regeneration media is essential for the successful recovery of putative transgenic sugarcane plantlets. The present investigation determines the eco-friendly cefotaxime concentration for the inhibition of *Agrobacterium* during co cultivation with sugarcane leaf disc explants. The disk diffusion assay with eight different concentration of cefotaxime viz; 0 (Control), 50, 100, 150, 200, 250, 300 and 350 mg /l were used in Yeast Extract Manitol (YEM) medium. *Agrobacterium tumefaciens* LBA 4404 strain harboring plasmid pBINAR, carried the selectable marker gene (*npt II*) for kanamycin. Out of these concentrations, cefotaxime @ 150 mg/l was found eco-friendly dose to suppress the growth of *Agrobacterium tumefaciens* and produced a vigorous shoots. Plant allows non-phytotoxic regeneration and survival rate was also good on this concentration. Consequently, the efficient protocol established in this study could be applicable for suppressing *Agrobacterium tumefactions* in further genetic transformation works of sugarcane.

Keywords: *Agrobacterium tumefaciens*, sugarcane, cefotaxime, yem

Introduction
Modern sugarcane varieties (*Saccharum spp*. hybrids) are economically important as a cash crop for producing sugar and energy in tropical and subtropical regions of the world. Both biotic and abiotic stresses affect the sugarcane cultivars. The improvement of sugarcane cultivars for sustaining quality is the major challenge for sugarcane breeders because of the highly heterozygous and polyploidy nature of this crop. Conventional breeding approach are time-consuming, it allows crossing of only closely related species rather limiting the diverse gene pool available for improvement. *Agrobacterium*-mediated transformation has several advantages in compare to other transformation methods such as the ability to transfer large pieces of DNA characteristic insertion into the recipient genome of a discrete segment of DNA at a low copy number and minimal re-arrangement of transferred DNA (Kumar *et al*. 2005; Tyagi *et al*. 2007) [6, 12].

Recently, the development of transgenic plants by genetic transformation has become an important tool for specific trait improvement. Transformation has been reported in sugarcane cultivar (Arencebia *et al*. 1998; Paulo A 2012; Wang *et al*. 2008) [1, 9, 14] with meager success. For the genetic transformation, desired explants are infected with *Agrobacterium* carrying gene of interest in an antibiotic free medium for 48-72 hrs. Successful transformation using *Agrobacteriuma* depends not only on explants type and plant regeneration system but also on the subsequent elimination of bacterium from co cultivated explants. The elimination of *Agrobacteriuma* is quite important because of long time presence of bacterium in shoot initiation media can cause problem in growth and development of transformed explants. The elimination of bacterium is usually achieved by adding one or more antibiotics to the culture medium (Matzk *et al*. 1996) [7]. Cefotaxime is a β-lactam antibiotic in the third-generation class of cephalosporin. It is a broad-spectrum antibiotic with activity against numerous gram-positive and gram-negative bacteria. Different concentration of cefotaxime has commonly been used as effective antibiotics for suppression of *Agrobacterium* in many plant transformation systems. Varghese *et al*. (2011) [13] had used cefotaxime @100 mg/l in black peapper. Cefotaxime and vancomycin were used @250 mg/l each as an antibiotics by Wiebke *et al*. (2006) [15] in Soyabean, Bernal *et al*. (2013) [3] had also used cefotaxime @250 mg/l in Indica rice transformation for the inhibition *of Agrobacterium*. Khan *et al*. (2013) [5] had used cefotaxime @1000mg/l as a pretreatment after co-cultivation in sugarcane for the elimination of *Agrobacterium*. Thus, cefotaxime had used by various workers at various doses in different crops during the past. Cefotaxime is a popular antibiotic in *Agrobacterium* mediated genetic transformation due to the eco-friendly nature. But their concentration varies from crop to crop. In sugarcane transformation, no more works are reported, that's why it indicates that

experimental parameters for reproducible sugarcane transformation have not been fully optimized. It is therefore essential to establish parameters for high efficiency regeneration and transformation of sugarcane plantlets.

In the present study we evaluate the effect of variable cefotaxime concentration on the elimination of *Agrobacterium* and their impact on successful regeneration of putative sugarcane transgenic plants. This information could be proved beneficial for successful transformation system in sugarcane to meet out the higher sugarcane and sugar production.

Materials and Methods

Antibiotics

Different concentrations of cefotaxime (0, 50, 100, 150, 200, 250, 300 and 350 mg/l) were used for evaluating growth suppression of *Agrobacterium* and frequencies of shoot formation and callus induction. The antibiotics cefotaxime were dissolved in water and filter sterilized with 0.22 µm nylon syringe filter. Sterilized cefotaxime were stored at -20 ^{o}C. Later, they were added to the autoclaved and partially cooled medium.

Agrobacterium strain and disc diffusion assay

Agrobacterium tumefaciens LBA4404 harboring plasmid pBINAR which carries the selectable marker gene (*npt II*) for kanamycin were used for the transformation experiment. Twenty four hours prior to transformation procedure, a single colony of *Agrobacterium* were grown in YEP liquid medium containing kanamycin @ 50 mg/l, streptomycin @ 250 mg/l and rifampicin @ 25 mg/l for 24 hrs at 28 ^{0}C on a rotary shaker @ 150 rpm until the culture O.D. reached 0.8-1 at 600 absorbance. The disc-diffusion assay was used to determine the impact of growth inhibition of *Agrobacterium* at 28 ^{0}C for 24 hrs by various concentration of cefotaxime. A sterile disc without antibiotic was used as control. The estimation of antibacterial activity was based on the areal diameter of the zone of inhibition formed around the disc. Petri plates were prepared by pouring 30 ml of YEP agar and letting solidify. Sterile discs with 6 mm treated with different concentration of cefotaxime were placed on center of YEP agar plats seeded with overnight culture of LBA 4404. Three replicates were conducted for each concentration of antibiotic.

Plant Material

Young whorl leaf rolls tissue (6-7 cm) of sugarcane variety CoS 08272 were collected from experimental field of Sugarcane Research Institute, Shahjahanpur, U.P. India. Leaf disc explants (8-10 mm diameter) were used as an experimental material. These leaf disc explants were cultured on MS medium having 3 mg/l 2, 4-D and wrap in aluminum foil for continues dark condition for 8-10 days (Pandey *et al*. 2011) [8]. After pretreatment, leaf disc explants were co cultivated with *Agrobacterium* and transferred to regeneration medium (0.2 mg/l BAP, 0.1 mg/l NAA and different concentration of cefotaxime) and incubated in light having 1600 lux for 16 hrs and rest 8 hrs in complete dark at 25±2 ^{o}C.

Results and Discussion

Impact of cefotaxime concentration on regeneration of plantlets

Agrobacterium-mediated genetic transformation depends on several factors like selection parameters under different antibiotic concentration, explants, co-cultivation parameters and regeneration media including hormonal concentration used for transformation. All these components contribute to an optimal performance in any transformation system and regeneration system. Here necrosis and slow regeneration of shoots were recorded after co cultivation of young whorl leaf disc explants with *Agrobacterium* having *Cry1 Ac* gene. Effect of BAP @0.2 mg/l in combination with NAA @0.1 mg/l was also evaluated with various concentrations of cefotaxime and without cefotaxime as a control. Results indicated that BAP @0.2 mg/l and NAA@ 0.1 mg/l in combination with cefotaxime @ 150 mg/l were found significantly higher shoot regeneration in sugarcane (Table 1, 2; Fig. 1, 3). The optimized shoot regeneration medium were also reported by Shujuan *et al*. (2017) [11] in woody plant by applying 0.03 mg/l 6-benzylaminopurine, 0.02 mg/l indole-3-butyric acid, and 0.0008 mg/l thidiazuron during *Agrobacterium*-mediated genetic transformation. Similar findings were also reported by Yanthan *et al*. (2017) [16] in *Drosera burmannii*. Concentration of cefotaxime with increasing order showed necrosis and leaves turned brown by moderate browning of leaf disc. The same finding with cefotaxime has also been reported earlier in strawberry by Haddadi *et al*. (2015) [4]. In *Agrobacterium* mediated genetic transformation, it is necessary to determine the right concentration of antibiotic to enable organogenesis and avoid escapes of the various concentrations, 150 mg/l of cefotaxime significantly produced a superior length and vigorous shoots, and also found an eco-friendly concentration for regenerated leaf disc explants sugarcane CoS 08272 and suppressing *Agrobacterium* (Table 1, 2; Fig. 1, 3). Similar findings reported earlier that cefotaxime @150 mg/l on shoot regeneration of strawberry leaf explants showed the good shoot regeneration (Haddadi *et al*. 2015) [4]. The same type of result was also published by Priya *et al*. (2012) [10] in Rice (*Oryza sativa L*.) transformation.

Disc-diffusion assay

Disc diffusion assay was taken to find out eco-friendly cefotaxime concentration for complete suppression of *Agrobacterium* after co-cultivation. Various concentrations of cefotaxime viz; 0, 50, 100, 150, 200, 250, 300 and 350 mg/l were used to find out *Agrobacterium* zone of inhibition. Inhibition zone was found enlarged with increasing concentration of cefotaxime (Fig. 2; Table 2). Minimum zone of inhibition with 5.55 mm diameter was recorded with cefotaxime @50 mg/l for *Agrobacterium* growth alone in disc diffusion assay. Significantly and maximum zone of inhibition (6.75 mm in diameter) was recorded at higher concentration of cefotaxime @350 mg/l but plant growth was suppressed simultaneously (Fig. 1, 2, 3; Table 1, 2). Based on this assay, experimental finding were concluded that inhibition zone for *A. tumefaciens* (LBA 4404) was significantly high but in another way high concentration of antibiotic were harmful to regenerated leaf disc explants of sugarcane variety CoS 08272. Similar findings were also reported by Asif *et al*. (2013) [2]. It was observed that, higher dose of antibiotics like cefotaxime inhibit cell wall synthesis and cause necrosis. Based on this assay, experimental finding were concluded that inhibition zone for *Agrobacterium* (LBA 4404) growth was significantly high and eco-friendly @150 mg/l which was most sensitive against cefotaxime without creating harmful stresses on transformed sugarcane plants (Fig. 1; Table 2). Thus, cefotaxime concentration @150 mg/l was emerging as valuable alternatives for suppressing *Agrobacterium tumefactions* with eco-friendly doses. This

concentration of cefotaxime could be re-emerging as valuable alternatives for suppressing *Agrobacterium* tumefactions with eco-friendly doses. Hence, the efficient protocol established in this study could be applicable for further genetic transformation in sugarcane.

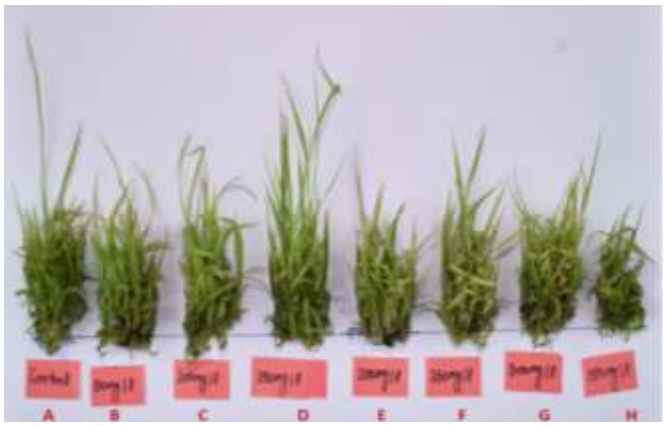

Fig 1: Effect of various concentration of cefotaxime on regenerated plantlets in *in vitro* conditions. Data were recorded per seven days interval and final data were recorded after three weeks. (A) Control (without antibiotic) (B) 50 mg/l (C) 100 mg/l (D) 150 mg/l (E) 200 mg/l (F) 250 mg/l (G) 300 mg/l (H) 350 mg/l

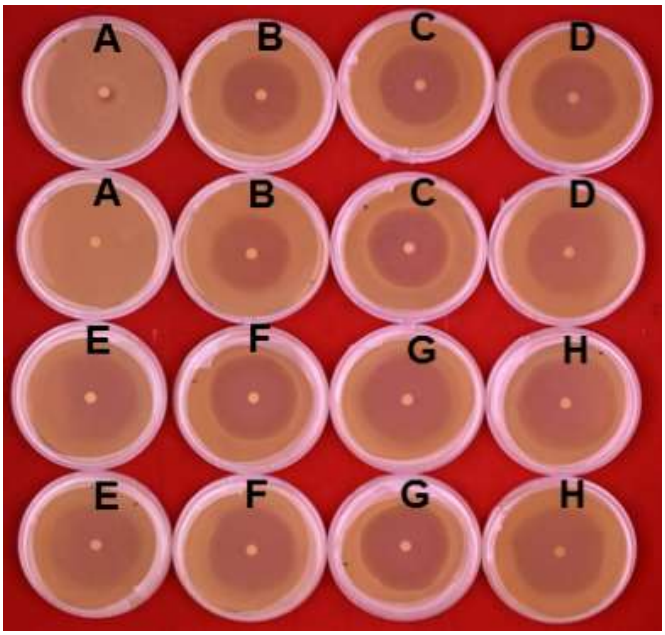

Fig 2: Disc diffusion assay of *Agrobacterium* at different concentration of cefotaxime. (A) 0 mg/l (Control) (B) 50 mg/l (C) 100 mg/l (D) 150 mg/l (E) 200 mg/l (F) 250 mg/l (G) 300 mg/l (H) 350 mg/l

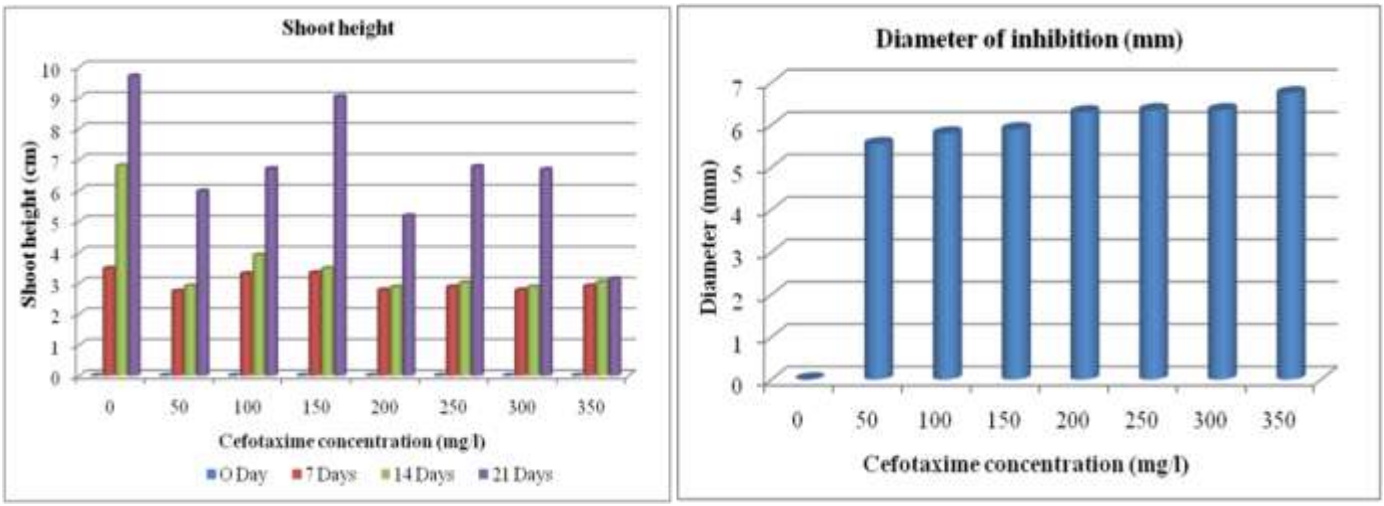

Fig 3: Effects of different concentrations of cefotaxime on shoot height and zone of inhibition

Table 1: Mean data of shoot number with various dose of cefotaxime

Sl. No.	Treatment	O Day	7 Days		14 days		21 Days	
			Shoot No.	SE ±	Shoot No.	SE ±	Shoot No.	SE ±
1	0 mg/l	6 Shoots	9.33	0.33	12.33	0.33	33.00	0.58
2	50 mg/l	6 Shoots	8.33	0.33	12.00	0.58	32.00	1.16
3	100 mg/l	6 Shoots	8.33	0.33	11.67	0.33	28.33	0.67
4	150 mg/l	6 Shoots	9.00	0.58	11.33	0.88	22.67	2.19
5	200 mg/l	6 Shoots	8.00	0.58	11.67	0.33	30.33	1.20
6	250 mg/l	6 Shoots	7.67	0.33	10.00	0.58	25.00	1.16
7	300 mg/l	6 Shoots	7.67	0.33	8.67	0.33	29.33	0.67
8	350 mg/l	6 Shoots	7.67	0.33	9.33	0.33	18.00	0.58
	C. D.	-	1.03	-	1.40	-	3.00	-
	SE (m)	-	0.37	-	0.46	-	0.98	-
	C. V.	-	7.06	-	7.27	-	6.21	-

Table 2: Mean data of shoot height and diameter of inhibition in cm with various dose of cefotaxime

Sl. No.	Treatment	Shoot height (cm)							Diameter of inhibition (mm)	
		O Day	7 Days	SE ±	14 Days	SE ±	21 Days	SE ±	Mean	SE ±
1	0 mg/l	2.5 cm	3.47	0.29	6.80	0.06	9.70	0.06	0.00	0.00
2	50 mg/l	2.5 cm	2.73	0.07	2.90	0.06	5.97	0.35	5.55	0.065
3	100 mg/l	2.5 cm	3.30	0.15	3.90	0.06	6.70	0.12	5.80	0.041
4	150 mg/l	2.5 cm	3.33	0.17	3.47	0.19	9.03	1.39	5.90	0.041
5	200 mg/l	2.5 cm	2.77	0.03	2.87	0.03	5.17	0.59	6.30	0.041
6	250 mg/l	2.5 cm	2.87	0.09	3.00	0.12	6.77	0.09	6.35	0.065
7	300 mg/l	2.5 cm	2.77	0.09	2.87	0.09	6.67	0.09	6.35	0.029
8	350 mg/l	2.5 cm	2.90	0.15	3.03	0.15	3.13	0.88	6.75	0.087
	C. D.	-	0.47	-	0.31	-	2.07	-	0.15	-
	SE (m)	-	0.15	-	0.10	-	0.68	-	0.05	-
	C. V.	-	8.86	-	4.90	-	17.62	-	1.85	-

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Conflict of interest
The authors declare no conflict of interest.

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