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Influence of antibiotics on regeneration efficiency in tomato

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Abstract

The protocol has been developed for an efficient regeneration and Agrobacterium mediated genetic transformation using antibiotics (100-400 mg/l) in cotyledon explants of tomato cvPKM-1. Among the three antibiotics viz, Cefatoxime (Cef), Timentin (Tim) and Carbenicillin (Cb) used along with the shoots induction medium (SIM – 0.1 mg/l IAA + 2.0 mg/l BAP). The antibiotics Cb and Cef showed the profound effect on the suppression of Agrobacterium tumefaciens growth at minimum concentration (200 mg/l). Whereas, Tim had shown its effect at 400 mg/l concentration. 100% explants survival was observed in all the concentrations of Tim tested in comparison to other antibiotics Cb and Cef used. Enhancement of shoots induction efficiency was also found at 400 mg/l Tim. The present investigation reports the effectiveness of Tim for regeneration and *Agrobacterium tumefaciens* mediated genetic transformation in cultivated tomato cv PKM-1.

Key words: Tomato; Cotyledon; Carbenicillin; Cefatoxime; Timentin; *Agrobacterium tumefaciens*.

Abbreviations: Cef_Cefatoxime; Tim_Timentin; Cb_Carbenicillin; SIM_Shoot Induction Medium.

Introduction

Genetic transformation in plants carried out by direct or indirect method to transfer gene of interest. Indirect method using *Agrobacterium tumefaciens* mediated genetic transformation is common and feasible to transfer gene of interest into different crop plants. It was two decades ago McCormick (1985) reported successful transformation in tomato. Since then many groups have reported the transformation ranging from 6% to 40% transformation efficiency (Park *et al,* 2003). However, it depends on many factors like genotype of the explants, explant position in the medium, regeneration ability of explants and also the impact of antibiotics used during transformation to eliminate *A.tumefaciens.*

The most commonly used antibiotics in genetic transformation experiments are Cefatoxime (Cef), Carbenicillin (Cb) and Vancomycin. However these antibiotics have showed a great negative impact on regeneration of transformed explants of tomato (Costa *et al,* 2000). Cef and Cb have a broad spectrum of activity against both gram positive and gram negative bacteria where they block the cell wall mucopeptide biosynthesis by inhibiting the cross linking of peptidoglycan by binding and inactivating

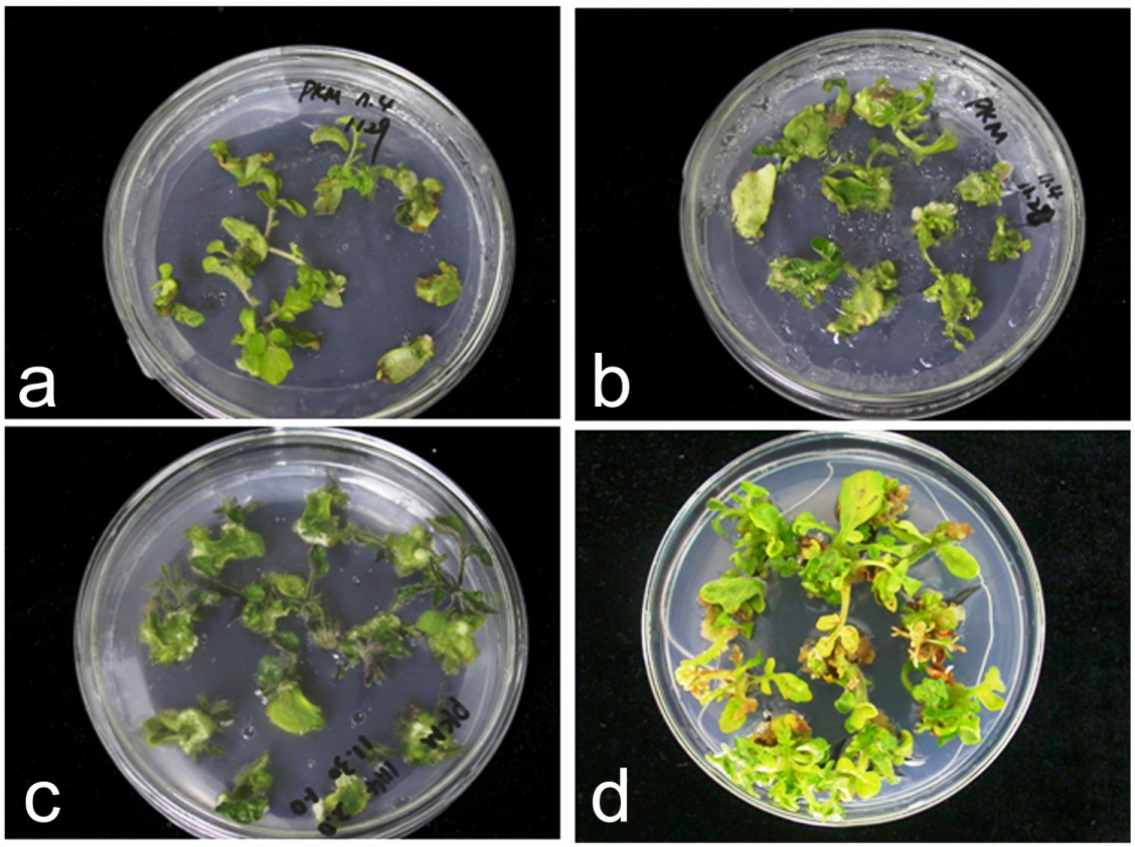


Fig 1. Effect of Timentin on regeneration of cotyledon explants of tomato cv PKM-1. **(a)** Cotyledon explants cultured on SIM + 100 mg/l Timentin **(b)** Cotyledon explants cultured on SIM+ 200 mg/l Timentin **(c)** Cotyledon explants cultured on SIM+ 300 mg/l Timentin **(d)** Cotyledon explants cultured on SIM+ 400 mg/l Timentin.

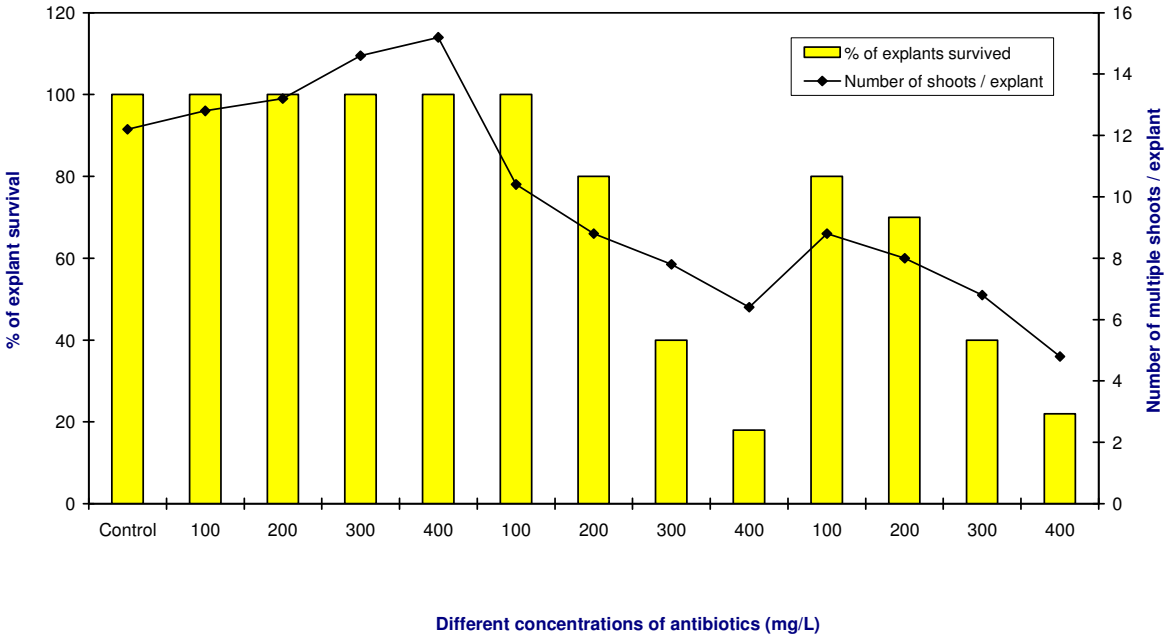
of transpeptidases. Timentin (Tim) a mixture of tricarcillin and clavulanic acid was reported to suppress *A.tumefaciens* in tomato genetic transformation (Costa *et al* 2000). In order to standardize the optimum concentrations of Cef, Cb and Tim on suppression of *A.tumefaciens* and also induction of multiple shoots production, a protocol was designed to screen the best antibiotic to eliminate the bacteria completely and also to promote the regeneration efficiency in tomato cv PKM-1.

Materials and methods

The seeds of tomato cv PKM-1 were obtained from Tamilnadu Agricultural University (TNAU), Coimbatore, India. The seeds were soaked under running tap water for 24 hours. These were sterilized with 70% (v/v) alcohol for one minute and followed by five minutes in 10% commercial bleach and 0.2% SDS followed by four to five rinses in sterile distilled water. Later the sterilized seeds were blot dried on sterile tissue paper and germinated aseptically on MS basal medium (Murashige and Skoog 1962). The pH of medium was adjusted to 5.8 with either 0.1N HCl or 0.1N NaOH and solidified with 0.8% (w/v) agar. The medium was sterilized in an autoclave at 121^0C under 15 psi for 15-20 minutes (ca.50ml medium/ flask). *In vitro* grown seedlings (7 days after germination) were used as the source of cotyledon (0.6 – 0.8 cm^2) explants.

To study the effect of antibiotics viz, Cb, Cef and Tim on regeneration efficiency, different concentrations of antibiotics (100 – 400 mg/l) individually was added to shoot induction medium (SIM) containing

Figure 2 : Effect of antibiotics on regeneration ability of tomato cv PKM-1



MS + 0.1 mg/l IAA + 2.0 mg/L BAP. For transformation experiments, the cotyledonary explants were submerged in bacterial suspension using cocultivation method. Present investigations were carried out using *A. tumefaciens* LBA 4404 harboring a binary vector containing AtNHX1 (antiporter gene). After cocultivation for three days, the explants were shifted onto SIM supplemented with different concentrations of antibiotics used. All the cultures were incubated at 25^0C under 16h photoperiod with light intensity of 40-60-umol^{-2}s^{-1} maintained by white fluorescent tubes.

Results

Cotyledon explants of tomato cv PKM-1 were used in the present investigations and the results are presented in Fig 2. All the antibiotics tested have completely inhibited the growth of *A.tumefaciens* at 300 mg/l (Fig 3).

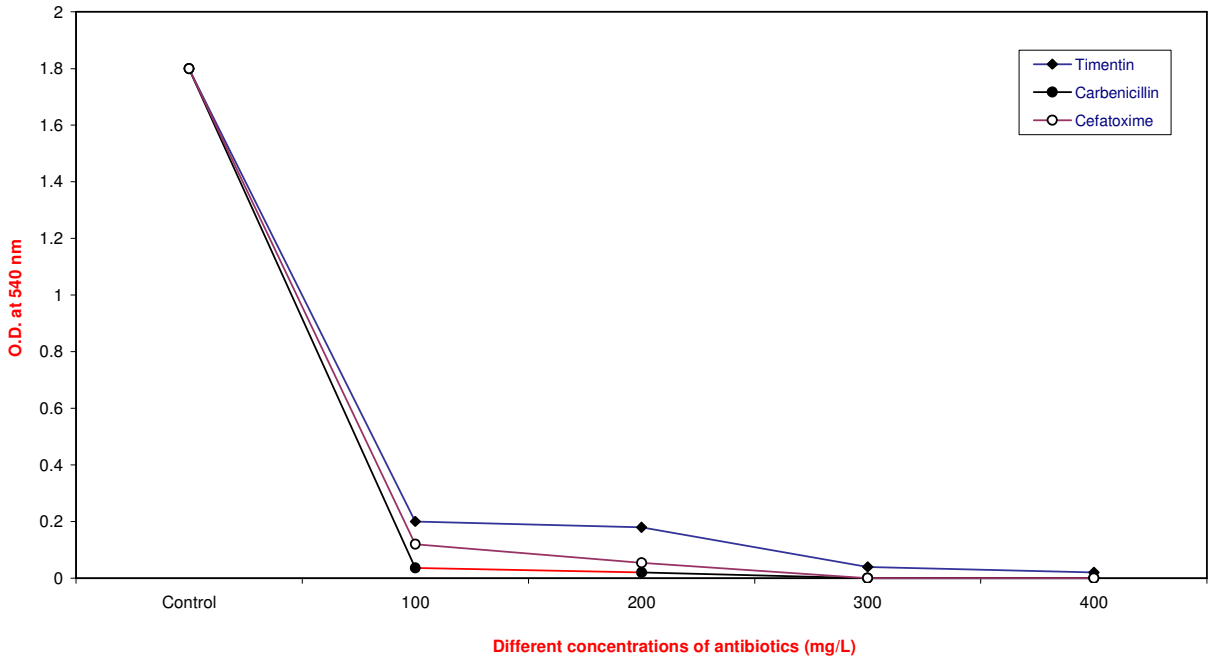
Effect of Timentin

The results on effect of Tim are presented in Fig 2. Various concentrations of Tim (100-400 mg/l) were added to SIM to determine the effect on regeneration ability from cotyledon explants. Absolute percentage of explant survival was observed in all the concentrations of Tim tested. As the concentration of Tim increased gradual increase in the induction of multiple shoots per explant was found. The highest number of shoots (16.2±0.32) formation per explant was observed at higher concentration (400 mg/l) of Tim compared to controls and other antibiotics tested (Fig 2). To know the effect of Tim on bacterial suppression, *A.tumefaciens* was grown on different concentrations of Tim (Fig 4) for 4 and 14 days. The bacterial growth was completely inhibited at 400 mg/l even after 14 days of culture on MS medium.

Effect of Cefatoxime

The effect of Cef (100-400 mg/l) was studied on cotyledon regeneration ability and bacterial suppression (Fig 2 & 4). The percentage of explant survival was reduced gradually from 100 to 400 mg/l Cef. Very less percentage (22%) of explant survival was observed at 400 mg/l Cef (Fig 2). Only two shoots per explant were induced at the same concentration of Cef. When *A.tumefaciens* cultured on MS with different concentrations of Cef, showed

Figure 3 : Effect of various antibiotics on the arrest of *Agrobacterium tumefaciens* growth



inhibitory effect on bacterial growth at 300 and 400 mg/l even after 14 days too (Fig 4).

Effect of Carbenicillin

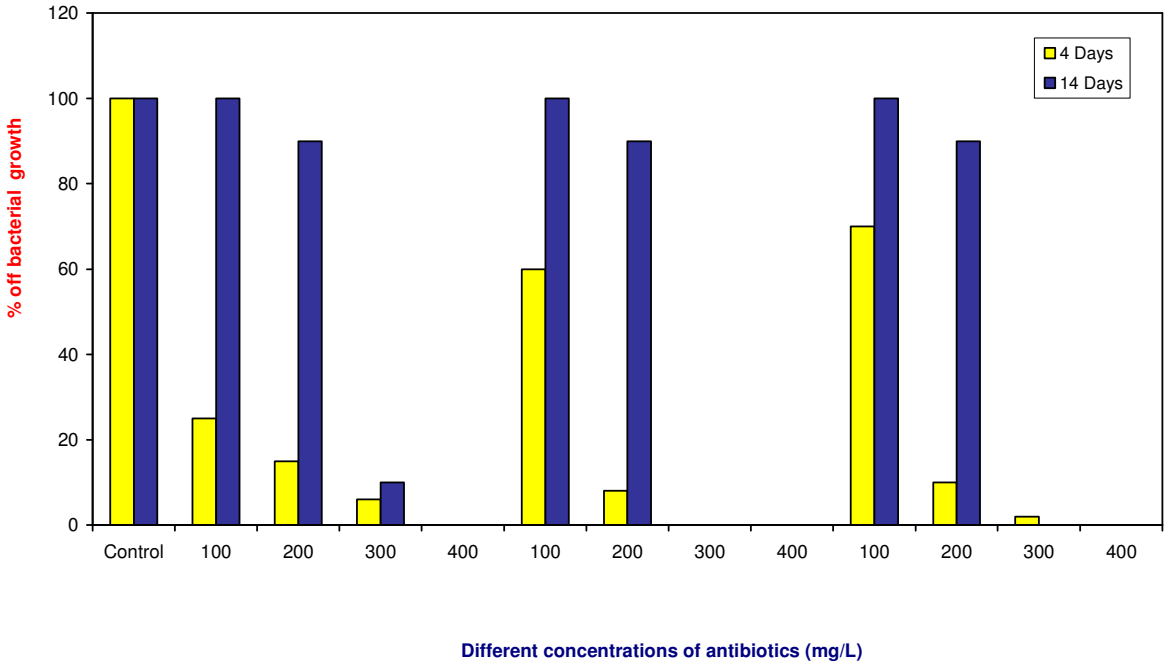
The data on effect of Cb on regeneration from cotyledon explants in tomato cv PKM-1 are presented in (Fig 2). Various concentrations of Cb (100-400 mg/l) were added along with the SIM. The percentage of explant survival was decreased from 100 to 400 mg/l Cb. Very less percentage (18%) of survival was recorded at 400 mg/l Cb. Number of shoots formation per explant was greatly reduced at 400 mg/l Cb (Fig 2). Total inhibition of *A.tumefaciens* growth was observed at 300 / 400 mg/l even after 14 days of culture on MS medium supplemented with Cb (Fig 4).

Discussion

Out of three antibiotics used, Cb had shown profound effect on suppression of *A.tumefaciens* growth at 300 mg/l (Fig 4) within 4 days of culture and it was found that the growth was resumed after two weeks if not sub cultured. However, at 400 mg/l it showed maximum bacteriostatic effect, even after two weeks of culture the bacterial growth was not observed (Fig 4). It was observed that at high concentration of all antibiotics used (400 mg/l) completely inhibited the growth of bacteria after 4 days and till two weeks.

The observation was made for two weeks as the subculture of regenerating explants was made for every two weeks in the current transformation experiments. Cef and Cb had shown bacteriostatic effect even at 300 mg/l whereas Tim had shown less percentage of colony formation of *A.tumefaciens* after 4 days (6%) and increased upto 10% within two weeks (Fig 4). Similar findings were also observed at low concentrations (100 and 200 mg/l) of Cef and Cb. From these observations it is concluded that the antibiotics at 400 mg/l have completely inhibited the growth of bacteria. Hence the effect of these antibiotics on regeneration of explants was also studied. The antibiotics Cb showed lethal effect at 400 mg/l where the regeneration ability was found to be less (3.4 shoots per explant). Similar observations were also recorded when Cef was used in the selection medium. At 400 mg/l Cef the cotyledon explants showed a poor response than Cb (Fig 2). Tim at low concentrations (100- 200 mg/l) was not

Figure 4 : Effect of various antibiotics on the arrest of *Agrobacterium tumefaciens* growth



effective in arresting the growth of *Agrobacterium,* but at 400 mg/l showed complete inhibition of *Agrobacterium* growth even in liquid and solid cultures too (Figs 3 & 4). Whereas Tim showed positive response at 400 mg/l in inducing more number of multiple shoots per explant (16.2 shoots/explant). Similar observations were also recorded by Cheng *et al,* (1998) on shoot regeneration of tobacco and Siberian elm and even for the suppression of *A.tumefaciens.* Costa *et al* (2000) have also reported an increase in the enhancement of multiple shoots formation from cotyledon explants of tomato cultivars at the concentration of 300 mg/l Tim. But in contrast to their results, we found that 300 mg/l of Tim + SIM doesn't suppress the *Agrobacterium* completely (Fig 2) and it needs subculture every week.

All the antibiotics tested, showed a negative effect on growth of *A.tumefaciens.* Cef in culture medium showed inhibition effect on multiple shoots induction at higher concentrations (Fig.2). Similar results were observed by Ling *et al ,* (1998) in tomato with the use of Cef. In the present investigations Cef showed the same results as that of Cb but profound callusing was observed at 400 mg/l Cef with decreased number of multiple shoots. In many transformation experiments the transformed cells of tomato were reported to be most sensitive to antibiotics in selective media (as selective media consists of two different antibiotics- one is used to eliminate *Agrobacterium* and other is to eliminate the untransformed cells).

An ideal antibiotic for inhibiting *Agrobacterium* species should be highly effective, inexpensive and very importantly without a negative effect on plant growth and regeneration (Cheng *et al,* 1998). Similarly in our observations we have found that Cb and Cef at all the concentrations tested (100-400 mg/l) had resulted a dramatic decrease in production of number of shoots per explant compared to control and Tim (Fig 2). Negative effects of Cef and Cb on induction of multiple shoots have already been reported in carrot and *Antirrhinum majus* (Okkels and Pedersen, 1998). It is advisable to use low concentrations of Cb and high concentrations of Tim for successful elimination of *A.tumefaciens* and also for induction of multiple shoots from the cotyledon explants in tomato cv PKM-1. Accordingly in our current transformation experiments we have used both Cb and Tim during first two weeks of culture

(data not presented). The concept of using two antibiotics is more effective for bacterial elimination (Horsch and King, 1983; Leifert *et al*, 1992) and Cef has been used, in conjunction with Vancomycin to eliminate effectively the bacteria in *Pinus pineas* (Humara and Ordas, 1999), *Citrus aurantifolia* (Pena *et al,* 1997) and several ornamentals (Leifert *et al,* 1992). In some of the plant systems a combination of three antibiotics was also used (100 µg/ml each of Cef, Cb and Mefoxin) to inhibit the growth of *A.tumefaciens* and efficient regeneration in apple leaf explants (Hammerschlag *et al,* 1995).

After screening antibiotics with Tim, Cef and Cb for phytotoxic effect and inhibiting the growth of *A.tumefaciens,* the SIM supplemented with 400 mg/l Tim has been found to be the efficient protocol for explant regeneration and *A.tumefaciens* mediated genetic transformation in tomato cv PKM-1.

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