

## Kanamycin sensitivity of cultured tissues of *Piper nigrum* L.

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### ABSTRACT

The kanamycin sensitivity for callus growth was studied *in vitro* in a cultivar of black pepper (*Piper nigrum*) using cotyledons as explants to investigate the suitability of kanamycin resistance as a selectable marker for *Agrobacterium* mediated transformation. Callus formation was completely inhibited at 50  $\mu\text{g ml}^{-1}$  and above concentrations of kanamycin suggesting that 50  $\mu\text{g ml}^{-1}$  is the minimum concentration needed to select the transformed tissues.

Key words : *Agrobacterium*, black pepper, callus, kanamycin sensitivity, *Piper nigrum*.

*Phytophthora* foot rot is the most serious disease of black pepper (*Piper nigrum* L.). Conventional breeding methods elude solution to this disease. Attempts are being made by using recombinant DNA techniques to incorporate resistance to this disease in black pepper. High rate of contamination in cultured tissues is also a limiting factor in *in vitro* studies of black pepper (Mathews & Rao 1984; Fitchet 1988).

Recombinant DNA technique to introduce foreign genes into plants involve the use of plant selectable markers such as kanamycin resistance. Kanamycin resistance is the most widely used selectable marker for plant transformation studies (Uchimiya, Handa & Brar 1989). Sensitivity of a particular plant

species or explant is important in developing a new transformation system employing the gene for neomycin phosphotransferase (NPT II) incorporating resistance to the antibiotic kanamycin. The purpose of the present study was to determine kanamycin sensitivity of callus growth of black pepper cotyledons used in the *Agrobacterium* mediated transformation.

Cotyledons of 3-4 month old black pepper cultivar Karimunda raised in sand filled basins served as the explant. Cotyledons were separated, washed thoroughly in running tap water and sterilized sequentially in i) 70% ethanol for 5 min followed by thorough washing in sterile water ii) 40% v/v sodium

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hypochlorite + few drops of Tween 80 for 5 min followed by thorough washing iii) 0.1%  $\text{HgCl}_2$  for 3 min. Finally, the cotyledons were washed thoroughly in sterile water, transferred to a sterile petriplate containing moist, sterilized filter paper and cut horizontally into segments of 0.2 cm  $\times$  1.5 cm size, after cutting off the four edges.

The cotyledon segments were cultured on a medium containing Murashige and Skooge salts and vitamins, sucrose 30 g  $\text{l}^{-1}$ , NAA 2 mg  $\text{l}^{-1}$  and 2, 4-D 1 mg  $\text{l}^{-1}$ . The medium was adjusted to pH 5.7 before autoclaving. Kanamycin (kanamycin sulphate) was filter sterilized and added @ 0, 10, 25, 50, 75, 100, 150 and 200  $\mu\text{g ml}^{-1}$  to the media dispensed as 25 ml per 100 ml conical flask and incubated at  $25 \pm 1^\circ\text{C}$  under 16 h photoperiod. There were four flasks per treatment with three cotyledon segments each and the whole experiment was repeated twice. The cotyledon segments were pre-incubated for 4 days on the same medium without the drug prior to transfer to the

medium containing the various concentrations of the antibiotic. Observations were recorded on contamination rate, callusing frequency and callus colour besides visually scoring the growth of the callus.

Callus formation was completely inhibited at 50  $\mu\text{g ml}^{-1}$  and above concentrations of kanamycin (Table 1). Callusing of the control explants started after 4 weeks of culturing, whereas it was delayed further by 2 weeks in the treatments containing kanamycin. Further, the control callus grew very vigorously as compared to the other treatments and it turned to green from the original white. The frequency of callusing of the control explants was also high (91.6 per cent) in the present study.

In case where kanamycin resistance is an efficient selectable marker, selection concentrations usually ranged from 20-300 mg  $\text{l}^{-1}$ . Transgenic tobacco are generally selected in 300 mg  $\text{l}^{-1}$  of kanamycin (Horsh *et al.* 1985) and

**Table 1. Response of black pepper cotyledons to various levels of kanamycin**

Kanamycin concentration ( $\mu\text{g ml}^{-1}$ )	% cotyledons callusing	Colour of callus	Callus growth
0 (Control)	91.6	White turned green	Vigorous
10	50.0	White	Slow
25	16.0	White	Slow
50	-	-	-
75	-	-	-
100	-	-	-
150	-	-	-
200	-	-	-

*Brassica juncea* cotyledons in 100 mg l<sup>-1</sup> (Mathews 1988). A kanamycin concentration of 20 mg l<sup>-1</sup> completely inhibited callusing from shoot tips of grape (Colby & Carole 1990).

Contrary to the earlier reports of high phenolic exudation and high rate of systemic infection observed in black pepper tissue culture (Mathew & Rao 1984; Fitchet 1988), the rate of contamination was reduced to 5 per cent in the study in all the treatments including control and there was no problem of phenolic exudation as well. The low rate of contamination in the present study might be due to the effect of the pre treatment protocol followed.

Kanamycin sensitivity of black pepper tissues was expressed as white calli up to 25 µg ml<sup>-1</sup> kanamycin followed by complete inhibition of callusing at 50 µg ml<sup>-1</sup> and above concentrations of the antibiotic in the present study. White callus has been reported previously in kanamycin sensitivity studies of *B. juncea* (Mathews 1988). Thus, 50 µg ml<sup>-1</sup> can be selected as the minimum concentration necessary to select *Agrobacterium* transformed calli from cotyledon explants of black pepper.

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