Effect of some antibiotics on the *in vitro* morphogenetic response from callus cultures of *Coryphantha elephantidens*

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Abstract

The effect of five antibiotics: carbenicillin, chloramphenicol, cefotaxime, kanamycin and hygromycin on the organogenesis from callus cultures of *Coryphantha elphantidens* (Lem.) Lem. have been studied. Carbenicillin and cefotaxime stimulated shoot regeneration from callus. All antibiotics under study suppressed rooting of *in vitro* formed shoots. After five sequential subcultures on kanamycin supplemented medium, antibiotic resistant callus was obtained. To study the impact of kanamycin on resistant callus, total protein content was also studied. Selected callus showed a remarkable increase in callus mass. Antibiotic resistant plants have been selected by screening callus pieces on kanamycin supplemented media. Total protein content increased with subsequent subcultures in kanamycin resistant callus. The kanamycin selected shoots withstood the stability test after 2 months on antibiotic free medium. Plants were raised from the callus, which formed roots in 20 mg dm^{-3} kanamycin, which was under study.

Additional key words: antibiotic resistant, elephant tusk cactus, *in vitro* selection, plant regeneration, plant tissue culture.

Introduction

Antibiotics are widely used in plant science to stimulate growth and development in tissue culture as observed in tobacco and carrot (Owens 1979, Chang and Schmidt 1995), tobacco (Pollock *et al.* 1983), barley (Mathias and Mukasa 1987), *Antirrhinum majus* (Holford and Newbury 1992), *Eleusine coracana* (Eapen and George 1990), *Betula pendula* (Valobra and James 1990) and *Panax ginseng* (Teng and Nicholson 1997). Phytotoxicity and stimulatory effect of antibiotics varies greatly with plant, explant type and even among different genotypes of the same plant species (Mathias and Mukasa 1987, Mathews 1988, Fiola *et al.* 1990). The ideal antibiotic should be soluble, stable, unaffected by pH and media components, lack side effects, broadly active, suitable in combination, inexpensive and non-toxic to plant cells (Falkiner 1988, 1990).

Members of family cactaceae can withstand high salinity and temperature in addition to their importance as ornamental plants (Johnson and Emino 1979, Bhau 1999, Wakhlu and Bhau 2000). Minocha and Mehra (1974) observed that the callus of *Neomammillaria prolifera* was resistant to high concentration of plant growth regulators, which are otherwise highly toxic to higher plants. No report is available on effect of antibiotics on such hardy and diverse plant group. This report investigates the effect of some antibiotics on the morphogenetic response of *Coryphantha elephantidens* and discusses the importance of antibiotics in *in vitro* morphogenesis. Paper also reports selection in callus cultures of *C. elephantidens* for resistance to kanamycin.

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Materials and methods

Tubercles (5 cm) of elephant tusk cactus [*Coryphantha elephantidens* (Lem.) Lem.] were taken, washed thoroughly with running tap water, surface sterilized in 70 % ethanol for 45 s, in 0.2 % $HgCl_2$ for 3 min and rinsed 5 times with sterile distilled water. Epidermis of tubercles was removed and the remaining tissue was sliced into transverse discs (10 mm in diameter). Disc produced thus was used as explants. MS (Murashige and Skoog 1962) basal medium containing 3 % sucrose and supplemented with different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (*Sigma Chemical Co.*, St. Louis, USA) were used for callogenesis and organogenesis. The medium was adjusted to 5.8 pH and gelled with 0.8 % agar (*Ranbaxy Lab. Ltd.*, Mumbai, India) prior to autoclaving. Cultures were incubated at temperature of 25 ± 2 °C, relative humidity of 50 ± 5 %, and irradiance of 32 μmol m^{-2} s^{-1} provided by cool-white fluorescent tubes for a 16-h photoperiod.

Callus growth was studied on MS medium supplemented with 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} kinetin and antibiotics in concentration 100 mg dm^{-3}. The fresh mass was determined in 5-d intervals. The initiation and regeneration media comprised of the MS basal medium supplemented with 2 mg dm^{-3} 2,4-D + 0.5 mg dm^{-3} kinetin and 1.5 mg dm^{-3} kinetin + 0.2 mg dm^{-3} 2,4-D, respectively. The effects of different antibiotics such as kanamycin sulphate (*Aries Pharmaceuticals*, Mumbai, India) (5, 10, 15, 20, 25, 50, 75 mg dm^{-3}), sodium cefotaxime (*Lupin Lab. Ltd.*, Bhopal, India) (50, 100, 250, 500, 1000, 2000 mg dm^{-3}), sodium chloramphenicol (*MAC Lab. Ltd.*, Mumbai, India) (100, 250, 500, 1000 mg dm^{-3}), hygromycin B (*Duchefa Biochemie BV*, Haarlem, The Netherlands) (5, 10, 15, 20, 25 mg dm^{-3}) and disodium carbenicillin (*Duchefa Biochemie BV*) (100, 200, 400, 600, 1000 mg dm^{-3}) on morphogenesis from callus cultures was studied. All antibiotics were filter sterilized and supplemented to the autoclaved medium. One callus piece (450 mg fresh mass) was used per treatment with 10 replications. The number of shoots produced per callus and shoot length were recorded. Shoot having minimum length of 1 cm were only taken into account.

To select the kanamycin resistant callus, it was subcultured after a regular interval of 4 weeks on the initiation medium supplemented with filter sterilized kanamycin. Two calli (250 mg fresh mass) per flask and 10 replicates per treatment was used. Callus fresh mass increase per flask after 4 weeks was determined, as reported by Meredith (1978).

Kanamycin resistant character of callus was assessed on the basis of visual observation of growth and discoloration. Total protein content in the kanamycin resistant callus was recorded at the end of each subculture. 0.3 M KOH hydrolysate of the callus was treated with 30 % trichloroacetic acid. The precipitate was dissolved in 0.1 M NaOH, from an aliquot of this solution protein was measured by the Folin phenol reagent (Lowry *et al.* 1951). Resistance stability of callus was tested by 2 methods. Firstly, by inducing organogenesis from callus cultures on kanamycin (20 mg dm^{-3}) supplemented medium after 2 months. Secondly, shoots formed were isolated and cut into transverse section for multiple shoot induction. Multiple shoots on these stem discs were induced on MS medium supplemented with 1.0 mg dm^{-3} kinetin. These shoots were again transferred on to MS basal medium supplemented with 20 mg dm^{-3} kanamycin for evaluation. Shoots were rooted on MS medium with 20 mg dm^{-3} kanamycin. Plantlets with well-developed roots were taken out of culture flask, washed in running tap water, and transferred to potting soil. Transparent polythene bags were used to cover them for ensuring high humidity during the first few days.

All experiments were repeated twice. Data was recorded after 4 weeks of culture initiation. Means of the replicates were compared using Duncan's new multiple range test (Duncan 1995).

Results

Morphogenetic evaluation: Callus cultured on carbenicillin, cefotaxime and chloramphenicol (100 mg dm^{-3}) supplemented media started growing slowly during 3rd week of culture. All antibiotics initially inhibited the callus growth. Kanamycin and hygromycin (100 mg dm^{-3}) completely inhibited the callus growth at all concentration (Table 1). Chloramphenicol, hygromycin and kanamycin at all concentrations inhibited formation of shoot primordia (Table 2, 3). Shoots formed on the kanamycin-supplemented medium lost their colour gradually and turned albino. At 100 mg dm^{-3} chloramphenicol favoured rhizogenesis from the callus. Kanamycin and hygromycin increased the anthocyanin pigment content in the callus, which was directly in contact with medium. Callus at the top and bottom turned pale yellow and ceased the growth.

Carbenicillin and cefotaxime stimulated shooting. Carbenicillin at 600 mg dm^{-3} raised the average shoot number from 11.5 to 17.7 shoots and average length from 3.4 to 4.9 cm (Table 2). Regeneration of shoots was inhibited at concentration of 1000 mg dm^{-3} or greater. Similar trends were observed when cefotaxime was added

to the medium. At a concentration of 500 mg dm^{-3} cefotaxime increased the average shoot number to 17.3 and shoot length to 5.2 cm (Table 2). Increase in average shoot number per callus by carbenicillin and cefotaxime was significantly different from other treatments. Regeneration response of callus on antibiotic supplemented medium was maintained for more than six months. At higher concentrations all antibotics proved to be highly toxic (Tables 2, 3). Cefotaxime at all concentrations favoured the callus formation, but carbenicillin favoured the callus growth upto concentration 400 mg dm^{-3}, beyond which it proved to be inhibitory to callus growth. Cefotaxime 500 mg dm^{-3} and carbenicillin 600 mg dm^{-3} when used without any growth regulators only gave rise to the friable pale green callus. It did not form any shoot primordia on the callus.

Table 1. Effect of different antibiotics (in concentration 100 mg dm^{-3}) on growth of *Coryphantha elephantidens* callus. Fresh mass [mg] was measured in regular intervals. Means ± SD, $n = 10$.

Antibiotic	0 d	5 d	10 d	15 d	20 d	25 d	30 d
Carbenicillin	500 ± 25	534 ± 38	584 ± 52	612 ± 55	667 ± 39	603 ± 27	567 ± 34
Hygromycin	500 ± 17	486 ± 29	427 ± 32	457 ± 21	473 ± 16	464 ± 11	433 ± 23
Chlorophenicol	500 ± 25	664 ± 41	759 ± 62	824 ± 48	915 ± 114	957 ± 74	977 ± 49
Cefotaxime	500 ± 22	467 ± 35	438 ± 11	408 ± 18	386 ± 9	374 ± 13	319 ± 19
Kanamycin	500 ± 15	467 ± 13	431 ± 16	391 ± 10	350 ± 11	283 ± 23	261 ± 12

Table 2. Effect of carbenicillin, chloramphenicol, and cefotaxime in different concentrations [mg dm^{-3}] on shoot number and length [cm] in 8-week-old callus cultures of *Coryphantha elephantidens* after 4 week of treatment. Means ± SD, $n = 10$. Means with the same superscript are not significantly different from each other at a 5 % level by Duncan's new multiple range test.

Concentration [mg dm^{-3}]	Carbenicilin number	length	Chloramphenicol number	length	Cefotaxime number	length
0	11.5 ± 0.9^{b}	3.4 ± 0.2	11.5 ± 0.7^{a}	3.4 ± 0.4	11.5 ± 0.7^{c}	3.4 ± 0.3
50	-	-	-	-	15.3 ± 1.9^{b}	4.5 ± 0.5
100	10.2 ± 1.1^{b}	3.6 ± 0.5	8.4 ± 0.9^{b}	2.9 ± 0.7	15.9 ± 2.1^{b}	5.0 ± 1.3
200	11.9 ± 0.4^{b}	4.2 ± 0.3	-	-	-	-
250	-	-	2.5 ± 0.3^{c}	1.3 ± 0.3	15.6 ± 1.5^{b}	4.7 ± 0.4
400	10.5 ± 1.0^{b}	3.2 ± 0.6	-	-	-	-
500	-	-	1.2 ± 0.7^{c}	1.0 ± 0.2	17.3 ± 2.4^{a}	5.2 ± 0.3
600	17.7 ± 1.3^{a}	4.9 ± 0.5	-	-	-	-
1000	6.8 ± 0.7^{c}	2.4 ± 0.3	0.0 ± 0.3^{d}	0.0 ± 0.0	8.6 ± 1.1^{d}	3.1 ± 0.5
2000	-	-	-	4.0 ± 0.3^{e}	-	1.3 ± 0.1

Table 3. Effect of kanamycin and hygromycin in different concentrations on shoot number and length [cm] in 8-week-old callus cultures of *Coryphantha elephantidens* after 4 week of treatment. At higher concentrations no shoots were formed. Means ± SD, $n = 10$. Means with the same superscript are not significantly different from each other at a 5 % level by Duncan's new multiple range test.

Conc. [mg dm^{-3}]	Kanamycin number	length	Hygromycin number	length
0	11.5 ± 2.2^{a}	3.4 ± 1.1	11.5 ± 2.5^{a}	3.4 ± 1.3
5	5.7 ± 1.4^{b}	1.3 ± 0.5	3.4 ± 1.2^{b}	1.6 ± 0.5
10	0.9 ± 0.3^{c}	1.1 ± 0.4	0.5 ± 0.0^{c}	1.0 ± 0.5
15	0.4 ± 0.0^{c}	1.0 ± 0.2	0.0 ± 0.0^{d}	0.0 ± 0.0

Shoots formed on the control medium were 100 % rooted in the MS medium supplemented with 0.5 mg dm^{-3} IBA and 0.5 mg dm^{-3} NAA. All the five antibiotics used, inhibited the rooting response from 100 % to below 30 % (data not presented). Chloramphenicol that favoured rhizogenesis on the callus showed no more significant difference in the improvement of rooting response from the in-vitro formed shoots.

Selection for kanamycin resistance: Callus cultures of *C. elephantidens* were maintained for seven-culture period on different (5, 10, 15, 20 mg dm^{-3}) concentrations of kanamycin. Growth of callus cultures on 0, 5 and 10 mg dm^{-3} kanamycin either remained same or increased during first cycle of selection. Kanamycin at concentrations 15, 20 and 50 mg dm^{-3} inhibited callus

growth (Table 4). Kanamycin at concentrations of 5 mg dm^{-3} or less did not inhibit the growth of a small part of the callus even after 4-week culture period. Growth inhibition of callus on kanamycin supplemented medium (15, 10, 20 mg dm^{-3}) increased from cycle 1 to cycle 3. The growth inhibition of that small bit of callus at 5 mg dm^{-3} kanamycin kept on declining from the cycle 1 onwards. For each cycle, the fastest growing, healthy, green sectors of callus were selected for transfer. But green sectors area decreased with each subsequent cycle.

Table 4. Effect of different concentrations [mg dm^{-3}] of kanamycin on the dry mass [mg] of resistant callus of *Coryphantha elephantidens* measured in regular intervals. Means ± SD, $n = 20$.

Concentration	0 d	5 d	10 d	15 d	20 d	25 d	30 d
0	598 ± 24.1	684 ± 34.6	941 ± 42.6	1359 ± 28.4	2189 ± 37.8	2354 ± 105.2	2457 ± 132.0
5	564 ± 23.7	583 ± 21.5	621 ± 25.4	753 ± 21.3	847 ± 17.3	883 ± 42.1	764 ± 27.4
10	512 ± 18.4	483 ± 13.7	507 ± 31.6	527 ± 25.3	559 ± 19.2	573 ± 26.3	582 ± 21.0
15	502 ± 27.2	475 ± 18.4	438 ± 23.6	408 ± 16.3	427 ± 23.7	459 ± 12.5	467 ± 22.6
20	486 ± 19.4	451 ± 14.7	404 ± 11.5	391 ± 10.2	352 ± 9.3	355 ± 11.4	368 ± 14.2

The total protein content of callus tissues estimated from different passages of culture revealed an increase in response to successive subculturing (Table 5). The increase of total protein content was seen in both control and antibiotic supplemented media, although the increase was much pronounced on the medium lacking kanamycin.

Table 5. Total protein content [mg g^{-1}(d.m.)] in calli of *Coryphantha elephantidens* during successive transfers on control and kanamycin (15 mg dm^{-3}) supplemented medium.

Subculture	1	2	3	4	5
Control	1	2	2	2	2
Kanamycin	6	8	10	14	22

During seventh cycle a green sector of callus tissue was isolated from cultures on 20 mg dm^{-3} kanamycin. This callus was further subcultured on kanamycin supplemented medium. Callus showed profuse growth on this medium. Callus formed 17 shoots on the organogenic medium. These shoots when multiplied *in vitro* formed 54 shoots. They were isolated and transferred on to the medium supplemented with 20 mg dm^{-3} kanamycin. These shoots showed normal growth in comparison to the shoots growing on the medium lacking kanamycin. Shoots were rooted on the medium fortified with 20 mg dm^{-3} kanamycin, 0.5 mg dm^{-3} IBA or 0.5 mg dm^{-3} NAA. Rooted shoots were hardened and transferred to the pots containing garden soil. They showed normal morphological features.

Discussion

The results present in this paper indicate that carbenicillin and cefotaxime promotes callus growth and morphogenetic response in callus cultures of *C. elphantidens*. These have been reported to produce breakdown product that gave physiologically active auxin phenylacetic acid (Holford and Newbury 1992). Cefotaxime has been reported to promote callus proliferation and morphogenesis also in apple leaf segments (Yepes and Aldwinckle 1994), wheat (Eapen and George 1990, Barret and Cassells 1994, Mathias and Boyd 1986, Borrelli *et al.* 1992), pearl millet (Pius *et al.* 1993) and carbenicillin in barley (Mathias and Mukasa 1987). It has been suggested by several workers (Borrelli *et al.* 1992, Holford and Newbury 1992, Nakano and Mii 1993, Teng and Nicholson 1997) that antibiotics promote morphogenetic response through their breakdown products formed by the metabolic activity of the cells during incubation period which mimic plant growth regulators. All five antibiotics inhibited the morphogenetic response and proves to be toxic for tissue, if incubated for longer times as earlier also reported in barley (Mathias and Mukasa 1986) and Sitka spruce (Sarma *et al.* 1995). These antibiotic products may degrade polyribosomes, inhibit protein synthesis and disrupt the membrane permeability (Zhang *et al.* 1999). Presence of kanamycin or hygromycin in the medium prevented chloroplast greening in the callus and shoot formed on the callus in *Rubus* (Joseph *et al.* 1990). This phytotoxicity varies greatly with plant type (Reed *et al.* 1995, Mathias and Mukasa 1987, Mathews 1988). Cefotaxime is a β-lactam antibiotic that inhibits cell wall synthesis in dividing bacterial cells. β-lactams binds to peptidoglycan transpeptidases, which are involved in the final stages of the cell wall synthesis and results in cell lysis (Selwyn 1980). Therefore, cefotaxime is likely to affect plant metabolism, which is supported by the results in this study. In the absence of growth regulators they did not induced morphogenetic potential. This suggests that

the antibiotic enhance the morphogenetic response, instead of inducing them. These results also suggests that in case of genetic transformation of *C. elephantidens*, kanamycin and hygromycin can be used to select transgenic plants.

The growth of sensitive callus was inhibited gradually with increasing concentration of kanamycin in the medium. Increase in total protein content after addition of antibiotics was recorded in *Oryza sativa* (Mukherji and Biswas 1985). A hypothesis to explain kanamycin induced callus growth is that the antibiotic increase the water content of callus which show a considerable fresh mass increase, and this could cause activation of protein synthesis and of metabolism in general. Most likely the kanamycin toxicity at high concentration is due to the action of the aminoglycosides on the ribosomes of chloroplasts and mitochondria. However, other possibilities of cell growth inhibition by the toxic levels of kanamycin cannot also be discounted (Pollock *et al.* 1983, Mathews 1988). Shoots growing and forming roots in the presence of kanamycin, showed the antibiotic resistance (Umbeck and Gengenbach 1983, Maliga *et al.* 1973, Gengenbach *et al.* 1977).

In conclusion, our results indicate that antibiotics can play an important role in enhancing the morphogenetic response of *C. elephantides*, although it differs from plant to plant under investigation. The mode of action involved in the effect of antibiotics on differentiation in plants still remain obscure. Prolonged exposure of callus to kanamycin for over six repeated cycles without loss of its acquired capability of increased tolerance to the antibiotic suggests that the induced mutation might take place at genetic level. This is further supported by its normal growth and root formation on the kanamycin supplemented medium.

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