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INFLUENCE OF KANAMYCIN ON EXPLANT AND CALLUS OF MOTH BEAN

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SUMMARY

Various tissues and explants of *Vigna aconitifolia* (Jacq.) Marechal cv. RMO-256 grown *in vitro* were incubated in the presence of a selectable marker antibiotic, kanamycin sulfate. Among the various tissues/ explants, leaves showed maximum tolerance to high levels of kanamycin (up to even 500 µg/ml). Shoot growth and root induction were affected severely and root induction was completely inhibited at 50 µg/ ml kanamycin. Callus tissues were found to be highly susceptible to kanamycin and callus induction reduced up to 50 per cent at 20 µg/ml levels. Low levels of kanamycin (20, 50 µg/ml) had a promotory effect on shoot bud morphogenesis in callus cultures. Higher levels (200-500 µg/ml) however, inhibited regeneration completely. Promotory effects of kanamycin were not observed in case of direct regeneration from leaf explants. Direct regeneration was completely inhibited at 100 µg/ml kanamycin.

Key words: **Callus, explant, kanamycin, moth bean, regeneration.**

INTRODUCTION

Moth bean (*Vigna aconitifolia* Jacq.) is the most drought tolerant pulse crop grown only in India. It is cultivated as *kharif* crop in Rajasthan, Gujarat, Maharastra and parts of U.P. and Bihar. The low yield potential is the major constraint for its production, which cannot be enhanced, as the genetic variability is very low. The unconventional approaches like genetic transformation may enhance the yield potential or other characters. *In vitro* regeneration system and requirement of a selectable marker are two conditions required for efficient exploitation of transformation. The commonly used selectable marker is kanamycin (Bhatia *et al.*1986). The phytotoxic effects of antibiotics on plant tissues have been studied by Young *et al.* 1984, Waldenmaier *et al.*1986. The success of transformation experiments depends on regeneration of whole plants from cells receiving the foreign DNA in the presence of toxic level of antibiotics. It has been demonstrated that the level of antibiotic tolerance and expression of sensitivity varies from plant to plant as well as in different explants of the same species (Mathews 1986). The present investigation was initiated with the objective to ascertain the tolerance and toxicity response of different *in vitro* grown explants to the kanamycin.

MATERIALS AND METHODS

The seeds of RMO-256 genotype of *Vigna aconitifolia* were used in the present investigations. Aseptic seedlings were obtained by inoculating surface sterilized seeds on B_5 basal medium. The immature primary leaf (145-150 mm^2) and apical bud (3-4 mm length) were excised from 6-8 days old, *in vitro* grown seedlings. Callus was obtained by incubating immature primary leaf explants on callus induction medium ($MS+B_5$ basal medium supplemented with 0.2 µg/ml kinetin + 7 µg/ml 2,4-D + 5 µg/ml NAA) at 28±2ºC under constant illumination (3000 lux). Primary callus once initiated from cut ends of leaf explants was subcultured at an interval of 15 days on callus induction medium. To observe the

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effect of kanamycin on seed germination, apical buds, leaf explants and callus growth, data were recorded after 15 days of incubation. In case of direct regeneration from leaf explants, observations were taken after 30 days and after 20 days of inoculation for indirect regeneration from callus cultures.

The effect of kanamycin on various explants (viz. primary leaf, apical bud and callus) was assessed by adding different concentrations of kanamycin to MS+B_5 medium. To study the effect of kanamycin on direct regeneration, immature leaf explants were incubated on MS+B_5 medium supplemented with 3 µg/ml BAP, with different concentrations of kanamycin. Frequency of regeneration response was calculated on the basis of 10 uniform explants. The indirect regeneration was assessed by incubating the callus culture on regeneration medium (MS+B_5 + 5 µg/ml kinetin + 1 µg/ml IAA) with different concentrations of kanamycin. Root and shoot length, biomass of seedlings, root induction frequency, biomass accumulation in apical buds, biomass accumulation in leaf explant and callus culture, were recorded to study the tolerance and toxicity response due to kanamycin. The data were analysed using standard statistical method i.e. DMRT.

RESULTS AND DISCUSSION

Seed germination was not at all affected by kanamycin but shoot length and root length were reduced and green pigmentation decreased with increasing levels of kanamycin (Table 1). At 200 µg/ml and above leaves turned yellow and shoots turned reddish indicating loss of chlorophyll. Similar results were also observed in *Brassica juncea* and *Vigna radiata* by Mathews (1986). This indicates sensitivity with increasing levels of kanamycin. It was noticed that root development was affected more although shoot development also decreased at high concentrations kanamycin in the medium, (Table 1). Roots were noticeably short and never produced any lateral branch. Similar observations were made in *Vigna radiata* (Mathews 1986) and *Lycopersicon esculentum* (Hille *et al.* 1986)

Shoot growth from apical bud was reduced at 50 µg/ml and higher levels of kanamycin. Unlike seedling

Table 1. Effect of kanamycin on apical bud explants, leaf explants and callus growth of *Vigna aconitifolia*.

Conc. of kanamycin (µg/ml)	*In vitro* apical buds (4-6 mm) cultured on MS + B_5* medium			*In vitro* leaf explants (145-150 mm^2) cultured on MS+B_5 medium		Stock callus cultured on MS+B_5 supplemented with Kn (0.2 µg/ml) 2, 4-D (7.0 µg/ml) & NAA (5.0 µg/ml)	
	Mean Shoot Length (mm)	% rooting in growing shoots	Biomass accumulation (mg)	Mean biomass (mg)	Explant size (mm^2)	Mean biomass (Fresh weight) (mg)	Morphological response
0 (Control)	73.0[a]**	100	61.6[a]	79.73[a]	271.20[a]	619.4[a]	Creamy-yellow
20	71.0[ab]	100	60.4[a]	76.93[b]	253.33[b]	440.2[b]	Creamy-yellow
50	67.2[b]	-	47.0[b]	74.60[b]	249.93[b]	362.0[c]	Creamy-yellow
100	57.2[c]	-	44.6[c]	68.73[c]	216.53[c]	361.0[c]	Creamy-yellow
200	39.0[d]	-	42.4[d]	67.27[c]	215.20[c]	320.8[d]	Creamy-yellow
500	28.0[e]	-	28.8[e]	62.27[d]	210.87[d]	291.0[e]	Creamy-yellow
SEm.	0.1257	-	0.5292	0.844	1.398	2.1441	

*MS + B_5 : Containing MS (1962) inorganic salts plus B_5 (Gamborg *et al.* 1968) organics, **Mean values followed by same letters do not differ significantly.

Abbreviation: Kn : 6- furfuryl aminopurine (Kinetin); 2, 4-D: 2, 4-dichlorophenoxy acetic acid; NAA: Nephthalene acetic acid.

development, however, root regeneration from apical bud explants was completely inhibited at 50 µg/ml and higher levels of kanamycin, (Table 1).

The inhibitory effect of kanamycin on auxillary and apical buds of *V. aconitifolia*, *V. radiata* and *V. mungo* was observed by Bhargava and Smigocke (1994) in gun mediated transformation experiment. They reported that rooting ability of putatively transformed apical meristems in the presence of kanamycin can become a simple screening technique for meristem carrying NPT II and associated genes. Mathews (1986) also made similar observation and suggested that the rooting of shoot explants in kanamycin containing medium was critical in selecting kanamycin sensitive plants.

Among the various explant systems (viz. seedlings, apical buds, leaf and callus) of *V. aconitifolia* tested for their susceptibility/tolerance to the kanamycin, leaf explants appeared to be most tolerant to kanamycin. Although leaf growth was affected by increasing levels of kanamycin but it was not completely checked even at 500 µg /ml level (Table 1). Contrary to this in *V. radiata*, Mathews (1986) observed that primordial leaves turned yellow at 100 µg/ml and above levels of kanamycin and completely bleached at 700 µg/ml kanamycin. Mathews (1986) further observed that Kanamycin did not affect green pigmentation in *V. radiata* seedlings and leaves were thick and green even upto 4000 µg/ml levels. This is, however, in contrast to observations made in present investigation where seedling pigmentation of *V. acontifolia* was reduced and at 200 µg/ml of kanamycin, leaves turned yellow and epicotyl and hypocotyl became reddish. Excised leaf explants did not show kanamycin toxicity even at 500 µg/ml level and but leaves from primordial seedling turned yellow probably due to nutrient deficiency caused by inhibitory effects of kanamycin on seedling roots, thus impairing their nutrient absorption capacity. This possibility is indirectly supported by the observation that kanamycin is probably transported in tissues to short distances. Young *et al.* (1986) have also observed that intact leaves were least affected by antibiotics. Such a growth stage specific response of an explant to a selectable marker antibiotic might permit selection of "escapes" along with "transformants" because in plant transformation experiments, green plants and shoots are generally selected as positive transformants having resistance to the selectable marker antibiotic (Viegas *et al.* 1987).

In the current investigation, it was observed that callus growth was drastically reduced even at low levels (20 µg/ml) of Kanamycin. Similar observation has been made in *Nicotiana piumbagnifolia* cell cultures where kanamycin and other aminoglycosides were found to be highly toxic at low levels of 10 µg/ml (Pollock *et al.* 1983). The lower levels of kanamycin (20-50 µg/ml) on *V. aconitifolia* had a promotory effect on shoot bud regeneration from callus cultures. However, regeneration frequency sharply declined at 100 µg/ml and above. Camus & Lance (1955) were the first to report that an antibiotic can stimulate the growth of normal plant tissue *in vitro*. Later, Owen (1979) reported a stimulation of the regeneration capacity in tobacco and carrot tissue by kanamycin. Biswas *et al.* (1985) reported some cytokinin like action of penicillin in carrot callus tissues Mathias and Boyd (1986) also have reported that Carbenicillin and especially cefotaxime could improve the regeneration capacity of tissue cultures of wheat at 60 µg/ml and 100 µg/ml concentrations.

Although low concentration of antibiotics has been reported to be promotory to morphogenesis but higher concentrations are inhibitory. For instance, in present investigation, regeneration in callus cultures sharply declined at 100 µg/ml kanamycin and completely inhibited at 200 µg/ml and above. Thus in "transformation-regeneration" experiments involving callus cultures of *V. aconitifolia* 200 µg/ml levels of kanamycin could be more useful for selection of transformants (Table 2).

The study showed that kanamycin had an inhibitory effect on direct regeneration from leaf explants even at 50 µg/ml level and regeneration was completely inhibited at 100 µg/ml and above, but 50 µg/ml kanamycin was promotory to regeneration in callus cultures and excised leaf explants did not show any toxicity symptoms even at 500 µg/ml kanamycin (Table 1). It has been demonstrated that the toxicity of kanamycin to plant material is dependent on the explant used (Okkels and Pederson 1988). Hille *et al.* (1986) also reported complete inhibition of regeneration from leaf discs in the presence of 5 µg/ml bleomycin. Among three *Vigna* legumes, *V. aconitifolia* has been proved to be highly

Table 2. Effect of kanamycin on seed germination and on direct regeneration from leaf explants and indirect regeneration from callus cultures of *Vigna aconitifolia.*

Conc. of kanamycin (µg/ml)	Seeds of cultivar RMO-256 cultured on MS + B_5 medium			6-8 days old leaf explants (145-150mm^2) were cultured on MS + B_5 supplemented with BAP (3.0 µg/ml)		Leaf derived callus was cultured on MS + B_5 supplemented with Kn (5.0 µg/ml l) & IAA (0.1 µg/ml)	
	% germination (15 days)	Mean shoot length (mm)	Mean root length (mm)	Regeneration response (Mean No. of shoots) (30 days)		Regeneration response (Mean No. of shoots) (30 days)	
				Frequency	Number	Frequency	Number
0 (Control)	100	157.80[a]	119.56[a**]	100	3.4 (2-4)a***	80	6.2 (5-7)a***
20	100	98.40[b]	46.52[b]	100	3.4 (3-4)a	100	7.2 (6-8)a
50	100	62.96[c]	21.80[c]	80	1.2 (1-2)b	100	7.2 (6-9)a
100	100	62.48[c]	19.60[d]	-	-	40	2.0 (1-3)b
200	100	60.88[d]	17.80[de]	-	-	-	-
500	100	58.00[e]	16.02[e]	-	-	-	-
S.Em.	-	0.0529	0.0647	-	-	-	-

*MS + B_5 : Containing MS (1962) inorganic salts plus B_5 (Gamborg *et al.*, 1968) organics; **Mean values followed by same letters do not differ significantly; *** Values in parenthesis represent range

Abbreviation: Kn : 6- furfuryl aminopurine (Kinetin); BAP: 6- benzyl aminopurine; IAA: Indole-3- acetic acid

morphogenic (Bhargava, 1991) and among various explants, immature leaf explants have been found to be endowed with a prolific regeneration capacity (Bhargava and Chandra 1989). Thus, leaf explants of *V. aconitifolia* attain a special significance in transformation protocols and this investigation suggests that for screening of leaf explants, regenerated NPT II carrying shoots, the level of kanamycin in the selection medium should be 100 µg/ml.

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