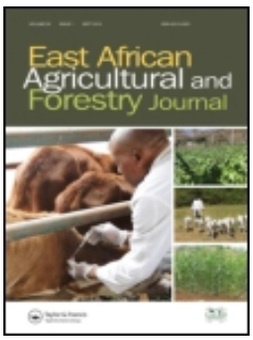

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# EFFECTS OF GENOTYPE AND *IN VITRO* MEDIA COMPOSITION ON MICROPROPAGATION OF SUGAR CANE

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The use of plant tissue culture for plant regeneration and propagation on large-scale requires a quantitative approach to optimised conditions associated with vegetative plant regeneration (Lal and Lal, 1990). Numerous factors affect morphogenesis and proliferation rates in micropropagation systems. These include the media composition (Nitsch, 1969; Huang and Murashige, 1977); the nature of donor and ex-plant (Gukasyan *et al.*, 1977; Wang and Ma, 1978); light (Hasegawa *et al.*, 1973); temperature (Carew and Staba, 1965; Seabrook and Cumming, 1978); polarity (Blotch, 1943, Ziv *et al.*, 1970); gas phase (Beasely and Eaks, 1979; Street, 1979), subculture (Hill, 1967; Chen and Galstron, 1967); genotype (Pierik *et al.*, 1974; Pierik and Steegmans, 1976; Seabrook and Cumming, 1978) and season (Robb, 1957; Nitsch and Hughes, 1978). In order to optimise plant regeneration and propagation, a wide range of factors should be investigated.

The genotype of the plant chosen for propagation might influence the response in tissue culture. Within a species certain genotypes appear to propagate easily while others fail to respond (Hughes, 1986). Genotype-specific effects on response to tissue culture have been reported in *Anthurium andraeanum* (Pierik *et al.*, 1974) and *Anthurium scherzerianum* (Pierik and Steegmans, 1976). Strong genotypic influence was also found in the tissue culture of wild oats, *Avena sterilis* and *A. sativa* (Rines and McCloy, 1981). Results from later studies led to the suggestion that screening genotypes and selection might lead to improvement in propagation capabilities in oats and recalcitrant cereals (Rines and McCloy, 1981).

Establishment of callus culture of sugar cane was reported by Nickell (1964) and regeneration of plants from callus was achieved much later (Barba and Nickell, 1969; Heinz and Mee, 1969). The most comprehensive study of media suitable for callus initiation and maintenance, shoot and root induction was conducted by Larkin (1982). Irvine *et al.* (1983) investigated other chemicals as possible substitutes of dichlorophenoxyacetic acid (2, 4-D) for callus initiation and maintenance and found picloram to be the only one to compete in effectiveness. Concentrations of 2, 4-D in the range of 2 to 7 mg $L^{-1}$ is suitable for callus maintenance. An approach that uses a mathematical model to determine the 2, 4-D and BAP ratios optimum for initiation and growth of callus was reported by Sauvaire and Galy (1980). Their calculation indicated maximal concentration ratios of 2, 4-D and BAP of 7.4 mg $L^{-1}$ to 9.3 mg $L^{-1}$ for root and 7.0 mg $L^{-1}$ to 4.7 mg $L^{-1}$ for shoot formation. In general, a sound rationale for choosing most tissue culture media is still lacking. There are considerable latitudes in permissible concentration of auxin (Maretzki, 1987).

Varieties of sugar cane grown currently are; complex hybrids synthesised from *Saccharum officinarum,* the noble canes (2n = 80), *S. barberi* (2n = 82, 116, 124), the Indian sugar canes; *S. sinense* (2n = 82, 116, 124), the Chinese cane and 2 wild species, *S. spontaneum* (2n = 54, 60, 64, 104, 112, 1128, 120) and *S. robustum* (2n = 80). The genes for sucrose accumulation are derived from *S. barberi*, *S. sinense* and *S. officinarum.* The wild cane, *S. robustum* and *S. spontaneum* have contributed genes for disease resistance, tolerance to environmental stress and higher yield through biomass production (Sreenivasan *et al.*, 1987). *Saccharum robustum* germplasm is limited to commercial varieties in Hawaii. Given such a broad genetic base from which commercial clones of sugar cane have

been developed, genotypes could have an influence on the tissue culture of sugar cane. For commercialisation of tissue culture for propagation purposes and elimination of important pathogens spread through the conventional method of propagation, there is need to have optimised tissue culture conditions for specific elite clones in order to cut down costs per seedling and to make the technology feasible for production of seedlings for farmers. This study investigated the influence of genotype and *in vitro* media composition on the tissue culture of 16 elite varieties with the aim of developing optimised protocols for commercial production of seedlings for sale to farmers.

## MATERIALS AND METHODS

### Plant Material

A standard variety Co421 (Co—Coimbatore, India) and elite locally bred-clones EAK 70-97 (East African), EAK 71-183, EAK 71-402, EAK 69-47, KEN 82-847 (Kenyan), KEN 82-216 and 8 introductions—Co945, Co331, Co617, Co1148, Co6415, N14, CB:38-22 (Campos, Brazil) and Phil 5460 (Philippines)—were included in the study. The newly released clones EAK 71-402, EAK 70-97, EAK 69-97 and Co945 have been reported to outyield the standard variety Co421 (Anon, 1994). Phil 54-60 and Co6415 are early maturing, KEN 82-216 is tolerant to *Striga asiatica*, while KEN 82-847, N14 and CB 38-22 are promising clones being evaluated for yield and adaptability (Anon, 1994).

### Tissue Culture

The tissue culture media contained basal medium components plus sucrose (30 g $L^{-1}$), myo-inositol (100 g $L^{-1}$), pyridoxine, HCl (1 mg $L^{-1}$), thiamine HCl (0.2 mg $L^{-1}$), nicotinic acid (1.0 mg $L^{-1}$), glycine (0.04 mg $L^{-1}$) and Difco-bacto-agar (7g $L^{-1}$) (medium containing all these components was designated MS1). The effects of 2, 4-D and genotypes on callus induction and growth were determined in a split-plot experimental design that was replicated 5 times. The main plots consisted of MS1 media with variable 2, 4-D concentrations—0, 1.5, 3 and 4.5 mg $L^{-1}$—while subplots consisted of 6 genotypes—Co1148, Co421, Co331, EAK 71-183, EAK 71-402, EAK 69-47.

The tissue culture medias were autoclaved at 121 °C and pressure of 15 p.s.i. for 15 min. Leaf petioles for callus induction were surface sterilised in 0.5% sodium hypochlorite for 15 min and rinsed 3 times in sterile deionised water. The petioles were sliced into 3-mm long sections with sterile forceps and cultured in media containing different 2, 4-D concentrations. The cultures were incubated under artificial light with 18-h photo-period at 26-27 °C. Callus fresh weights were determined after 6 weeks of *in vitro* culture. In a previous experiment, preliminary results indicated highly significant main effects due to 2, 4-D and genotype, and interactions between 2, 4-D and genotypes. This experiment was redesigned to focus on simple effects and include more genotypes. A split-plot design with 4 replications was used. The mainplots consisted of MSI media with 2, 4-D at 1.5 and 3 mg $L^{-1}$, while subplots consisted of 10 genotypes (Co1148, Co331, Co617, Co945, Co6415, Co421, EAK 71-183, EAK 71-402, EAK 69-47 and EAK 70-97). Callus fresh weights were determined after 8 weeks of *in vitro* culture.

Somatic embryogenesis and regeneration of plantlets with shoots and roots was achieved on MS1 media supplemented with 10-15% (v/v) filter-sterilised coconut water. Effects of cytokinin on further multiplication of seedlings were investigated in a completely randomised block design (CRBD) with 10 replications. The treatments included 6-benzylamino-purine (BAP) added to MS1 media at (0, 2, 3 mg $L^{-1}$). Effects of indole-3-butyric acid (IBA) (at 0, 2, 3 mg $L^{-1}$) on root growth among seedlings was investigated in a CRD with 10 replications. Treatments consisted of additions of the above concentrations of IBA or Naphthalene acetic acid (NAA) to MS1 media. Root and shoot development were determined after 4 weeks of *in vitro* culture. Seedlings with developed shoots and roots were transplanted into pots and then into the field for further evaluation. Preliminary experiments on regeneration of KEN

TABLE I - ANALYSIS OF VARIANCE FOR FRESH WEIGHTS OF 6 CLONES CULTURED IN MEDIA CONTAINING 2, 4-D (AT 0, 1.5, 3 AND 4.5 mg/l) DETERMINED FOR 1 TO 6 WEEKS

| Source | | Mean squares | | | | |
|---|---|---|---|---|---|---|
| | df | Week 1 | Week 2 | Week 3 | Week 4 | Week 6 |
| Blocks | 4 | 41.90ns | 32.36ns | 135.82ns | 494.59 | 549.50ns |
| 2,4-D | 3 | 94.65ns | 4662.96** | 37927.04** | 217724.94** | 1559221.60** |
| Error (a) | 12 | 44.77 | 64.76 | 66.06 | 213.84 | 691.42 |
| Genotypes (B) | 15 | 83.41* | 429.67** | 5659.70** | 60242.74** | 15723.59** |
| AXB | 45 | 17.53ns | 59.38ns | 439.92** | 4489.41** | 14703.86** |
| Error (b) | 80 | 26.49 | 44.62 | 113.66 | 281.04 | 405.62 |
| Total | 119 | | | | | |

*—Significantly (P = 0.05) different, **—highly significantly (P = 0.01) different, ns—not significantly (P = 0.05) different

82-247 KEN 82-247, KEN 82-216, N14, Phil 5460, CB 38-22 also produced plantlets.

## RESULTS AND DISCUSSION

### Effects of 2, 4-D on Callus Development

The influence of 2, 4-D on callus induction and development from leaf petioles of 6 clones studied from week 1-6 was highly significant (P = 0.01) at weeks 2, 3, 4, 5 and 6 (Table I). Mean separation of callus fresh weights determined at week 6 using protected least significant difference (LSD) showed that the 4 levels of 2, 4-D differed significantly (P = 0.05) in inducing and sustaining callus development (Fig. 1). 2, 4-D at 3 mg $L^{-1}$ decreasingly produced the most callus growth followed by 1.5 and 4.5 mg $L^{-1}$. No callus was produced in medium without 2, 4-D (0 mg $L^{-1}$) but the leaf tissues expanded slightly and turned green. However, since the interactions between genotypes and 2, 4-D were significant, the genotypes did not behave in the same way over each level of 2, 4-D concentration and vice-versa. Interpretation based on main effects could be inconsistent and therefore erroneous. It was therefore important to consider simple effects discussed later.

### Effects of Genotype on Callus Development

The effect of genotypes on callus induction and development using four 2, 4-D treatments, determined for 6 weeks, were highly significant (P = 0.01) at weeks 1, 2, 3, 4 and 6 (Table I). There were significant (P = 0.05) differences between mean callus fresh weights for the 6 genotypes at week 6. Genotype EAK 69-47 produced the heaviest callus followed by Co6415, Co331, Co617, EAK 71-402 and EAK 71-183 (Fig. 2). The genotypes did not, respond uniformly to the different concentrations of 2, 4-D in the media because of significant interaction between 2, 4-D and genotypes. It was thus necessary to consider simple effects of genotypes over 2, 4-D levels.

### Simple Effects of Genotypes over 2, 4-D Levels

To investigate simple effects, callus was induced from 10 genotypes at 2 levels of 2, 4-D —1.5 and 3 mg $L^{-1}$—and the experiment replicated 4 times. The mean calli fresh weights produced at 2, 4-D concentration of 3 mg $L^{-1}$ were significantly (P = 0.05) heavier than those produced at 1.5 mg $L^{-1}$ for all the 10 genotypes except for Co617 and Co945 (Fig. 3). Clones EAK 69-47 produced

the highest amount of callus at both levels of 2, 4- D, while clones Co421 and EAK 70-97 produced the lightest calli fresh weight at levels 1.5 and 3 mg $L^{-1}$. Although clones Co617 and Co945 produced more callus at 3 than 1.5 mg $L^{-1}$ 2, 4-D, the difference in callus fresh weights were not significant. For propagation purposes, it would be more economical to induce clones Co617 and Co945 into calli at 1.5 mg $L^{-1}$ and the other 8 clones at 3 mg $L^{-1}$.

The preceding differential response among the 10 genotypes to 2, 4-D treatments could be due to the difference in genetic constitution among the clones. For example, EAK 69-47 parentage is Co798 (Melting pot), whose pedigree traces include *Saccharum spontaneum* (India, 2n = 64), Saretha group (*S. barberi* 2n = 90, 92); Kansar (*S. sinense*) and Black Cheribon (*S. officinarum*) (Tew, 1987). Clone Co617 parentage includes *Saccharum spontaneum* (India, 2n = 64), Stripped Mauritius, Saretha group (*S. barberi*) (2n = 90, 92); Kansar (*S. sinense*) and Black Cheribon (*S. officinarum*) (Tew, 1987). The proportion of the genome contribution by the different species varies with method of breeding, number of backcrosses, choice of donor and recurrent parents. Whereas the nature of the differences in the genotypes might be unknown, in petunia lines, only a few genes (oligogenic) control differences in hormonal requirements for callus induction between genotypes (Izhar and Power, 1977). In *Petunia hybrida,* at least 2 genes controlled cytokinin requirements (Hanson *et al.,* 1978). At the biochemical level there are several reasons why genotypes modulate their response to callus induction in media containing 2, 4-D, a phenoxyalkanoic herbicide that at low concentration (0.022 to 22 mg $L^{-1}$)

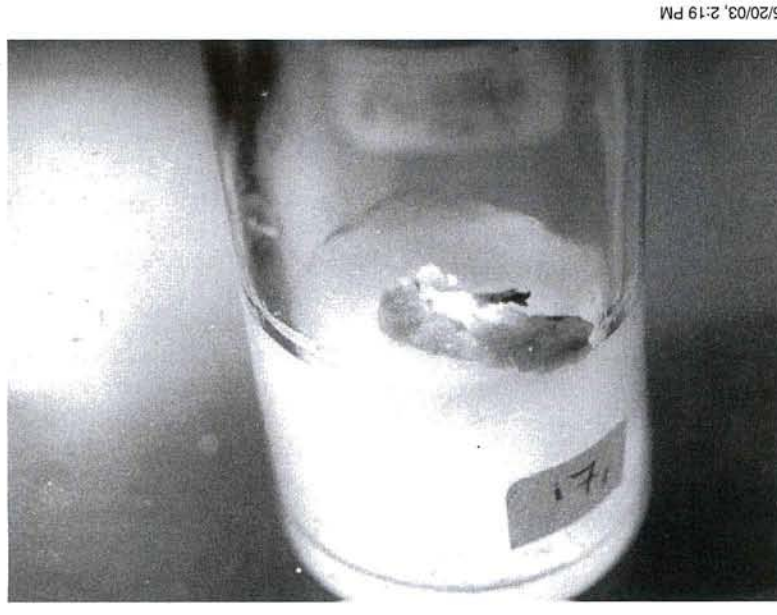


**Fig. 1—Callus induction from leaf explants after 133 days of *in vitro* culture in MS1 media containing 3 mg/L of 2, 4-D**

**Fig. 2—Somatic embryogenesis of callus after 36 days of *in vitro* culture in MS1 medium containing 10% coconut water**

**Fig. 3—Development of callus into plates with shoots and roots in clone Co1148 after 50 days culture in MS1 medium containing 10% coconut water**

**Fig. 4—Field evaluation of tissue culture produced clone Co331 aged 6 months**

induces cell division and enlargement. Cell enlargement is due to increases in activities of autolytic and synthetic enzymes that affect cell wall plasticity and synthesis of new cell wall materials (Cleland, 1971; Alberstain, 1976; Loos, 1977). Like any other herbicide, its effectiveness depends on the concentration of the active ingredient that reaches the active sites of biochemical action. This in turn depends on inter-related processes including efficiency of uptake, translocation within the plant and accumulation at the active sites. At any of these stages 2, 4-D might be degraded to non-active metabolite and rendered immobile (Kirkwood and Fletcher, 1968). It is also possible that the sugar cane clones studied might differ in their ability to metabolise 2, 4-D. Conjugation of phenoxyalkanoic herbicides with glucose, amino acids or proteins has been reported (Feug *et al.*, 1975, Chkanikov *et al.*, 1977). Formulations of 2, 4-D with glucose esters occur in tissue section or cell cultures of several species (Chkanikov *et al.*, 1977). Conjugation with amino acids occurs in a number of species such as aspartic acid-2, 4-D conjugate (Feug *et al.*, 1975) and glutamic acid-2, 4-D conjugate (Chkanikov *et al.*, 1972). Amino acid conjugates might not necessarily cause inactivation of 2, 4-D.

In the current study, levels of 2, 4-D of 4.5 mg $L^{-1}$ resulted in significant decreases in callus fresh weights (Fig. 1). This was contrary to observations made by Maretzki (1987) that 2, 4-D concentrations of 2 to 7 mg $L^{-1}$ were suitable for callus maintenance. The discrepancies in suitable ranges of 2, 4-D for callus induction and maintenance could be attributed to the differences in genotypes. At low concentrations, 2, 4-D primarily influences nucleic acid metabolism by stimulating the RNA polymerase activity subsequent RNA and protein synthesis, whose products are required for cell division and enlargement. However, at high concentrations, phenoxyalkanoic herbicides act as uncouplers and inhibitors of oxidative phosphorylation (Kirkwood, 1976; Moreland, 1980). The fine structure of mitochondria and other epidermal cells of *Rhea decolour* and *Eichhornia crassipes* has been reported to be affected by 2 4-D (George, 1971).

**Regeneration of Plantlets from Callus**

Sugar cane plantlets were regenerated from callus through somatic embryogenesis (Plates 1-4). Ability for callus to develop into plantlets was influenced by composition of MS media and genotype. The MS media that was not supplemented with 10-15% water did not support somatic embryogenesis. The number of plantlets regenerated per callus increased linearly ($Y = 22.71 + 1.33X$, $r = 0.78$, $r^2 = 0.61$) with callus fresh weights (Fig. 4); EAK 69-47 produced the heaviest callus and the most plantlets per callus, EAK 71-402 produced the least callus and the fewest plants per callus. Calli from certain genotypes such as Co1148 were hard to develop into plants even when cultured in MS media supplemented with coconut water. The specific requirement of certain genotypes for specific media and growth condition is not unusual. Thus, media should be optimised for specific genotypes, both for commercial purposes and regeneration of genetically engineered clones for maximum number of regenerants.

Genotype-specific influences on callus induction and development have been reported in *Anthurium andraeanum* (Pierik *et al.*, 1974) and in *A. scherzerianum* (Pierik and Steegmans, 1976). Only 1/3 of the *A. andraeanum* and 3/4 of *A. schezerrianum* genotypes formed callus and subsequently produced plants.

The mean number of plantlets that multiplied per subcultured plantlet increased linearly ($Y = 8.2 + 0.97X$, $r = 0.86$, $r^2 = 0.74$) with callus fresh weights (Fig. 4). Genotypes that produced heavier calli also had higher ability to proliferate into plantlets when subcultured in multiplication media.

## CONCLUSIONS AND RECOMMENDATIONS

Concentration of 2, 4-D at 3 mg $L^{-1}$ was optimum for callus induction in clones Co945, Co421, Co6415, Co331, EAK 71-183, EAK 71-402, EAK 69-47, EAK 70-97, while 1.5 mg $L^{-1}$ was optimum for clones Co617 and Co1148. There was reduced response in callus induction at 4.5 mg $L^{-1}$ of 2, 4-D. Somatic embryogenesis was achieved using media with filter-sterilised coconut water. Further multiplication of plantlets was achieved in media with 2 mg $L^{-1}$ BAP, while root production was realised in media with IBA or NAA at 2 mg $L^{-1}$. Genotypes that produced the heaviest calli also consistently produced more plantlets per callus and proliferated more in multiplication media than genotypes that were poor callus producers. Preliminary results on genetic stability of tissue culture produced plantlets as assayed biochemically using isozyme analysis has showed somaclonal variability ranging from 4% in genotype Co331 to 22% in genotype EAK 69-47.

## SUMMARY

Fifteen introductions and locally bred elite sugar cane (*Saccharum genus*) clones—Co945, Co617, Co331, Co1148, Co6415, Co421, EAK 71-183, EAK 71-402, EAK 69-47, EAK 70-97, KEN 82-847, KEN 82-216, N 14 (Natal, South Africa), Phil 5460 and CB 38-22 were cultured into plantlets from leaf explants in Murashige and Skoog (MS) basal medium. Callus was induced from leaf ex-plants in MS media supplemented with dichlorophenoxyacetic acid (2, 4-D) at 1.5, 3 and 4.5 mgl-$^{1}$. Effects of 2, 4-D on callus fresh weights was highly significant ($P = 0.01$). Effects of genotypes on callus fresh weights and interaction between 2, 4-D and genotypes were significantly ($P = 0.05$) different. Simple effects of 8 genotypes—Co945, Co421, Co6415, Co331, EAK 71-183, EAK 71-402, EAK 69-47, EAK 70-97—at 2 levels of 2, 4-D (1.5 and 3 mg $L^{-1}$) were significant ($P < 0.05$). The 8 genotypes produced significantly more callus at 3 mg $L^{-1}$ of 2, 4-D than at 1.5 mg $L^{-1}$. Genotype EAK 69-47 produced the heaviest callus fresh weight followed by Co6415. Genotypes Co617 and Co1148 did not produce significantly heavier callus at 3 mg $L^{-1}$ than at 1.5 mg $L^{-1}$ 2, 4-D. Seedlings with shoots and roots were regenerated from callus in MS media supplemented with 10% (v/v) filter-sterilised coconut water. The number of plantlets regenerated per callus increased linearly ($Y = 22.71 + 1.33X$, $r^2 = 0.61$, $r = 0.78$)

with callus fresh weights. Further multiplication of seedlings was optimal in MS medium plus 2 mg $L^{-1}$ BAP, while root development was optimised at 2mg$L^{-1}$ IBA or NAA. The number of plantlets multiplied per sucker were linearly ($Y = 8.2 + 0.97x$, $r^2 = 0.86$) related to callus fresh weights.

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