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Full Length Research Paper

Sensitivity of some quantitative and yield characters of 'Egusi' melon *(Colocynthis citrullus* L.) to treatment with microtubule inhibitors

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A major constraint in the improvement of 'Egusi' melon lies in the fact that attempts at transferring desirable genes into the plant from wild relatives are limited by interspecific hybridization barriers. Therefore, antimitotic agents were used as a means of introducing genetic variability for possible incorporation into breeding programmes of the crop. Seeds soaked in water and three different concentrations (0.01, 0.001 and 0.0001 M) of oryzalin and colchicine were planted directly in field plots where data on some quantitative and yield characters were collected. Plants derived from seeds treated with 0.001 and 0.0001 M of both drugs had significantly longer leaves with larger areas, longer internodes and took a fewer number of days to flower with a higher fruit setting percentage than those soaked in 0.01 M and water, respectively. These plants equally produced bigger pod variants that resulted in the production of more seeds, indicating that they were probably polyploids, which was confirmed by flow cytometry. An examination of the plants in subsequent generations indicated that all of these traits were stably integrated and expressed in the mutants, tacitly implying that they were fixed and can be introgressed into traditional varieties through backcrossing for further improvement of 'Egusi' melon.

Key words: Antimitotic drugs, colchicine, mutations, oryzalin, pod variants, polyploids.

INTRODUCTION

'Egusi' melon (*Colocynthis citrullus* L.), is a highly drought tolerant annual cucurbit widely distributed in parts of West Africa. It is especially very common within the savanna and forest vegetation belts stretching from Cote d'Ivoire to Cameroon. Rarely cultivated as a sole crop, it is a non-climbing creeper which provides weed suppression in traditional crop husbandry when intercropped with maize, cassava and yam at the onset of the rainy season. The seeds, which are a good source of high quality edible oils (53%), protein (28%) and some important minerals (Oyolu, 1977), are a major soup ingredient in West Africa (Purseglove, 1991) where they are highly relished. The ground seeds are usually utilized as a thickener in the preparation of traditional soups. The oil extracted from the seeds is of high nutritional value as it is composed mainly of unsaturated fatty acids, which gives a unique flavour to foods during cooking. It is equally used for the production of pastries, margarines and soaps (Ajibola et al., 1990), and has recently been found to have enormous potential in the production of biodiesel (Giwa et al., 2010).

Lately, there has been a tremendous increase in the production of 'Egusi' melon, especially in Nigeria where it is cultivated over an area of 361,000 ha with a seed yield

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Abbreviations: DAPI, 4,6-diamino-dino-2-phenylindole; **ANOVA,** analysis of variance; **LSD,** least significant difference; **DNA,** deoxrybonucleic acid.

of about 347,000 ton per annum, probably because of the increased awareness of its economic value. In rural areas, for example, it is cultivated as a major economic activity for income generation. It is equally used in Nigerian folk medicine, especially in the south-western part of the country, where the seeds of a local variety “Barablack edge” serve as part of a decoction containing roots of some other herbs and onion for the treatment of gonorrhoea in women (Okoli, 1984). However, in spite of the many indications quoted for the use of ‘Egusi' melon there has hardly been any comprehensive study undertaken with the aim of improving either its productivity or overall agronomic performance because conventional breeding methods using wild rustic melon species in order to transfer desirable genes into the genus are limited as a result of interspecific hybridization barriers (Rhimi et al., 2006).

It is widely believed that, increasing the ploidy level confers distinct advantages for the development of important agronomic traits in plants such as larger and deeper coloured flowers in carnation (Yamaguchi, 1989) and cyclamen (Takamura and Miyajima, 1986), thicker and broader leaves that result in the production of larger fruits in apple (Solov’eva, 1990), a better adaptability of individuals and increased organ and cell sizes in *Andropogon gerardii* (Keeler and Davis, 1999) and a tendency for stomata cell size to increase in *Manihot esculenta* (Carvalho et al., 1999). Driven by the desire to improve the performance of ‘Egusi’ melon such that its current yield per hectare can be increased significantly, a major objective of the present study was to examine the extent to which some of its agronomic traits could be influenced following treatment with microtubule inhibitors such as oryzalin and colchicine, two known spindle toxins that have the ability to induce an increase in ploidy level of most plant species. In the current study our desire was to evaluate the role of these inhibitors in enhancing the variability of ‘Egusi’ melon by improving some of its agronomic characters and yield through an increase in the numbers of pods produced per plant as well as seeds contained in a pod in the drug-treated plants and those of the mutant generations.

MATERIALS AND METHODS

Plant material

Seeds of **“**Barablack edge”, a local variety of ‘Egusi’ melon (hereinafter simply referred to as ‘Egusi’) selected from a batch obtained courtesy of the National Horticultural Research Institute, Ibadan, Nigeria, were used for this study. All field experiments reported here were conducted at the research garden behind the Department of Genetics and Biotechnology, University of Calabar, Calabar, Nigeria during the dry season in 2005 and, thereafter, repeated annually until 2009.

Treatment with oryzalin and colchicine

‘Egusi’ seeds were soaked in water (control) and three different concentrations (0.01, 0.001 and 0.0001 M) of oryzalin and colchicine for 24, 48, 72 and 96 h, respectively, at room temperature. After the treatment of the seeds in all the stated concentrations and durations they were washed several times in running tap water to remove excess chemicals and planted directly in field plots following a randomized complete block design with 5 replications. Ten seeds were sown per stand at a spacing of 1.5 m between and 0.5 m within rows. These were observed daily until maximum seedling emergence was achieved about 5 days after planting at which point the survival percentage was recorded for each treatment. The seedlings were subsequently thinned to 2 per stand after about a week of germination when they were fully established.

The following quantitative and/or morphological characters of the plants derived from the control seeds and those treated with the different levels of antimitotic agents were recorded at the appropriate growth stages: (i) frequency of seedling emergence, (ii) percentage (%) seedling survival, (iii) leaf length and area at flowering, (iv) number of leaves per plant at flowering, (v) number of branches per plant at flowering, (vi) length of internodes at flowering, (vii) vine length at flowering, and (viii) number of flower buds. The productivity of the plants derived from the different treatments with respect to the number of pods per plant, pod weight as well as number of seeds per pod was equally recorded at fruit maturity. These seeds were replanted to evaluate the production of the first mutant populations (M_1) that were again subjected to a series of rigorous selections between 2007 and 2009, which resulted in the production of second, third and fourth mutant generations (M_2 – M_4 plants).

At the end of the fourth generation, plants derived from the original parents treated with various levels of oryzalin were examined critically and compared with those derived from the M_1, M_2 and M_3 generations to determine if some important morphological and yield characters were stably fixed and expressed in the mutants.

Flow cytometry analysis

Leaves of the untreated control, plants derived from the initially oryzalin-treated seeds and the mutants derived from these lines were chopped with a razor blade into an ice-cold neutral buffer. The samples were filtered through a 30 mm mesh and stained with 4,6-diamino-dino-2-phenylindole (DAPI). Flow cytometric analysis was performed with a ploidy analyzer (Partec PA) according to Ntui et al. (2009).

Statistical analysis

The quantitative data collected were subjected to analysis of variance (ANOVA) test in order to determine the level of significance among means. Significant differences among group means were determined using the least significant difference (LSD) at the level of $p< 0.05$.

RESULTS

The germination percentage of seeds treated with distilled water (control experiment) ranged between 95 and 100%. Regardless of the soaking duration, both oryzalin and colchicine appeared to have very little effect on number of days to seedling emergence and survival percentage when treated at low concentrations, as it took only a few days for such seeds to emerge. However,

Table 1. Mean (± SE) values of number of days to emergence and survival frequency of 'Egusi' melon seedlings exposed to varying concentrations of oryzalin and colchicine after 48 h of treatment.

Agronomic response	0.0001 M	0.001 M	0.01 M	0 M (Control)
Days to seedling emergence				
Oryzalin	$4.47^{ab} \pm 0.24$	$5.33^{b} \pm 0.23$	$7.17^{c} \pm 0.83$	$4.07^{a} \pm 0.18$
Colchicine	$5.07^{ab} \pm 0.18$	$7.18^{c} \pm 4.24$	$12.07^{d} \pm 0.18$	$4.08^{a} \pm 0.12$
Survival percentage				
Oryzalin	$79.00^{a} \pm 3.66$	$78.07^{a} \pm 5.56$	$56.18^{b} \pm 7.36$	$81.89^{a} \pm 4.29$
Colchicine	$78.21^{a} \pm 0.22$	$68.18^{b} \pm 1.26$	$20.96^{c} \pm 4.20$	$82.23^{a} \pm 2.24$

*Means followed by the same case letter within a given row indicate no significant difference at 5% probability.

these parameters tended to decline with increasing concentrations of oryzalin and, especially colchicine, when treatment duration was extended beyond 24 h. For example, seeds treated with 0.01 M solution, which was the highest concentration of either antimitotic agent used in the current study, took a longer period to emerge. Such seedlings, especially those treated with colchicine, equally had a lower survival percentage (Table 1). Interestingly, while there were no significant differences ($P > 0.05$) in the percentage of seedling emergence between seeds soaked in 0.001 and 0.0001 M solutions of oryzalin and colchicine, respectively; however, this was significant ($P < 0.05$) when seeds were soaked in 0.01 M concentration of both chemicals even though, overall, seedlings derived from seeds treated with this level of oryzalin emerged faster and performed better than those treated with the same level of colchicine. Thus more plants survived the effects of 0.01 M concentration of oryzalin than those treated with the same level of colchicine (Table 1).

Generally, plants derived from oryzalin treatment responded better than those with colchicine at all the concentrations investigated. The effect of oryzalin and colchicine treatments on plant length, leaf length, internode length, number of flower buds and number of pods per plant are presented in Figure 1. It was generally observed that seeds treated with different concentrations of oryzalin did not present any significant effect ($P > 0.05$) on the number of leaves per plant and length of vines; perhaps an indication that these parameters were not seriously affected by its treatment. However, irrespective of the antimitotic agent used, plants generally performed better from seeds treated initially with low concentrations for longer durations than at higher concentrations even for short periods of treatment. For example, oryzalin and colchicine induced a higher number of flower buds as well as early flowering variants at low concentrations (0.0001 M) when compared to those produced by the control plants and at high concentrations. At treatments with concentrations higher than 0.0001 M, the number of days required for the plants to flower also increased (data not shown).

An intriguing observation noted here was the fact that 'Egusi' plants derived from treatments with the lowest concentration (0.0001 M) of both chemicals, especially oryzalin, yielded about two pods per plant (Table 2) with an average combined weight of 8.9 kg. Conversely, plants produced from treatments with higher concentrations of the chemicals and the control (0 M) usually yielded only one pod, which weighed about 5.6 kg on the average. Furthermore, there were differences between the numbers of seeds produced by the different concentrations of the two chemicals. Generally, treatments with lower concentrations of both chemicals produced a higher number of seeds per pod than those produced using higher concentrations as well as the control treatment. For example, the results presented in Figure 2 show that there were differences in the influence of microtubule inhibitor concentrations used on the number of seeds produced per pod just as there were also differences observed in the number of branches per plant at flowering (Table 2).

It was speculative from these results that polyploidy may have been induced in these 'Egusi' variants with the bigger pods. To confirm this, a flow cytometric examination of leaf tissues taken from plants raised from seeds of the control treatment, those producing these bigger pod variants as well as the progenies derived from them was conducted. The results from these evaluations are presented in Figure 3. Most of the antimitotic agent-treated plants as well as the mutants derived from them were actually polyploids, with some of them being mixoploids (2n/4n or 4n/8n).

One of the primary objectives of the current study was to confirm if morphological and yield traits that had been improved following treatment with low concentrations of both oryzalin and colchicine could be stably integrated in the progenies derived from the treated seeds. Interestingly, as shown in Table 2, most of these characters were actually fixed and showed better performance in the mutants than the original parents and untreated control.

DISCUSSION

It is not surprising that seeds treated with very high concentrations of antimitotic agents, such as those used

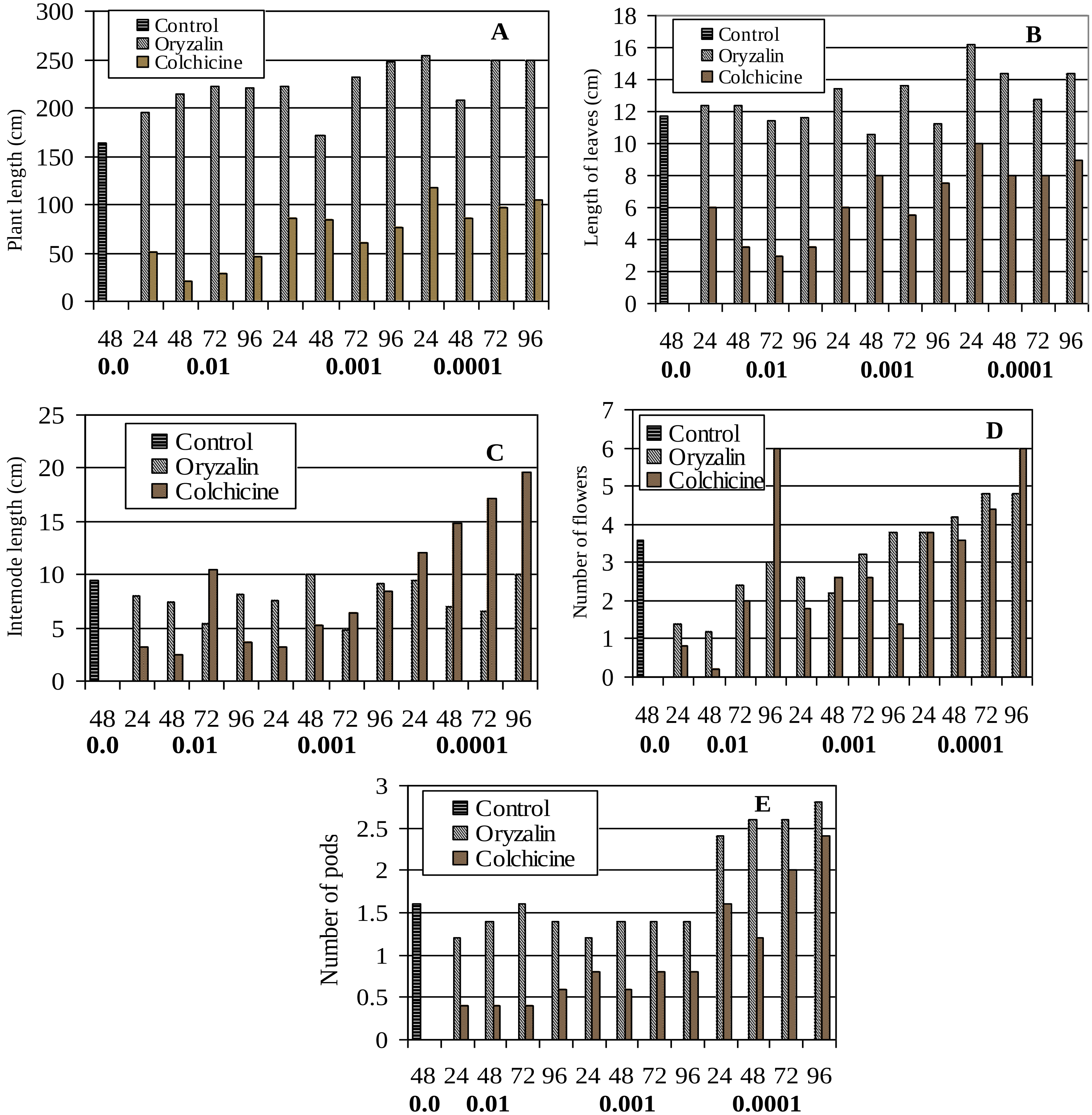

Figure 1. Response of "Egusi" melon plants to treatment with water (depicted here as 0.0 for 48 h) and 0.01, 0.001 and 0.0001 M, respectively, of oryzalin and colchicine after 24, 48, 72 and 96 h of soaking: (A) length of plant, (B) length of leaves, (C) internode length, (D) number of flowers, and (E) number of pods per plant.

in the current study, took a longer period to emerge. It is equally not surprising that such seeds or even the seedlings derived there from, especially following colchicine treatment, had a lower survival percentage, as they usually died before or just a few days after germination. In accordance with data already reported by Blakeslee and Avery (1937), Forni-Martins and Cruz (1985), Tosca et al. (1995), Mensah and Akomah (1997)

Table 2. Mean (± SE) values of some morphological characters in 'Egusi' melon derived from control plants compared with those of oryzalin-treatment and three mutant generations.

Treatment of parent plants*	Plant generation	Plant length (cm)	Length of internodes (cm)	Number of branches per plant	Number of leaves per plant	Number of flowers	Number of pods
Control (0 M)	-	227.0±58.6	7.6±0.4	17.6±1.47	43.4±2.2	4.6±0.5	1.8±0.3
0.01 M	Parent	195.6±36.8	7.2±0.4	15.4±1.36	60.8±1.1	2.4±1.2	1.4±0.2
	M_1	171.6±29.9	7.0±0.6	14.9±1.74	55.2±2.0	2.4±0.2	1.2±0.2
	M_2	213.5±44.1	7.6±0.7	12.8±0.33	57.2±6.6	2.2±0.2	1.6±0.3
	M_3	218.3±8.9	8.6±1.9	16.2±0.32	59.5±6.2	2.4±0.6	1.4±0.6
0.001 M	Parent	222.2±28.3	8.4±0.2	17.9±1.9	54.6±5.9	3.6±0.5	1.6±0.2
	M_1	230.5±36.8	8.0±0.6	17.4±0.98	55.6±3.9	3.2±0.4	1.4±0.3
	M_2	231.6±40.3	8.4±0.5	18.2±0.36	54.2±0.3	3.2±1.2	1.4±0.3
	M_3	238.6±9.6	8.2±2.3	18.0±0.66	54.2±0.6	3.8±0.6	1.6±0.4
0.0001 M	Parent	227.4±22.1	10.4±1.4	20.8±2.02	44.8±0.49	3.9±0.9	2.8±0.2
	M_1	237.6±26.2	10.0±0.3	21.6±1.77	48.0±12.1	4.2±1.2	2.6±0.3
	M_2	248.6±9.6	12.8±0.2	20.9±1.12	46.6±8.0	4.8±1.6	2.4±0.3
	M_3	248.6±9.6	12.8±0.6	21.0±2.16	48.4±0.6	5.2±1.1	2.6±0.6

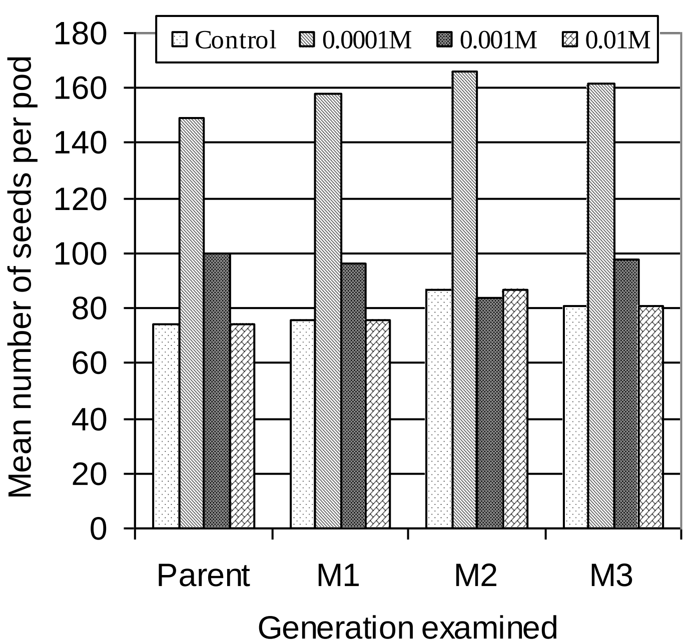

Figure 2. Effect of treatment of the initial seed lot with varying concentrations of oryzalin on the mean number of seeds produced per pod in the parent and three generations of mutant 'Egusi' melon plants.

and Mensah et al. (2005) in other plant species, the ability of high concentrations of antimitotic drugs such as colchicine and sodium azide to interact with chromosomal deoxrybonucleic acid (DNA) has been ascribed as the reason for the general toxic effects associated with their mutagenic properties (Mensah et al., 2007). It is, therefore, reasonable to speculate that the generally lower survival percentages seen with the colchicine-treated seeds in this study could be attributed mainly to either physiological disturbance, which possibly may have affected some biochemical pathways in the seedlings or this may have arisen as a result of chromosomal damages resulting from the toxicity associated with direct interaction between colchicine and DNA molecules.

Regardless of the exact explanation for the lower survival percentage seen with the seeds treated with the highest level of colchicine in the current study, it is possible that the early establishment of those treated with oryzalin could equally serve as a reason for the higher performance of such plants for all the morphological characters evaluated here. Apparently this development does not seem peculiar to 'Egusi' alone as prolongation of plant maturity following treatment with high concentrations of mutagenic agents has also been demonstrated in sesame (Mensah et al., 2007). In fact, early as well as late maturing variants have been isolated in various crops after treatment with mutagenic chemicals depending on the type and concentration of the chemical used.

Of immense agronomic interest and significance in the current study was equally the fact that 'Egusi' plants derived from treatments with very low concentrations, especially of oryzalin, yielded an increased number of pods with a higher combined weight per plant compared to all other treatments that produced just a single pod.

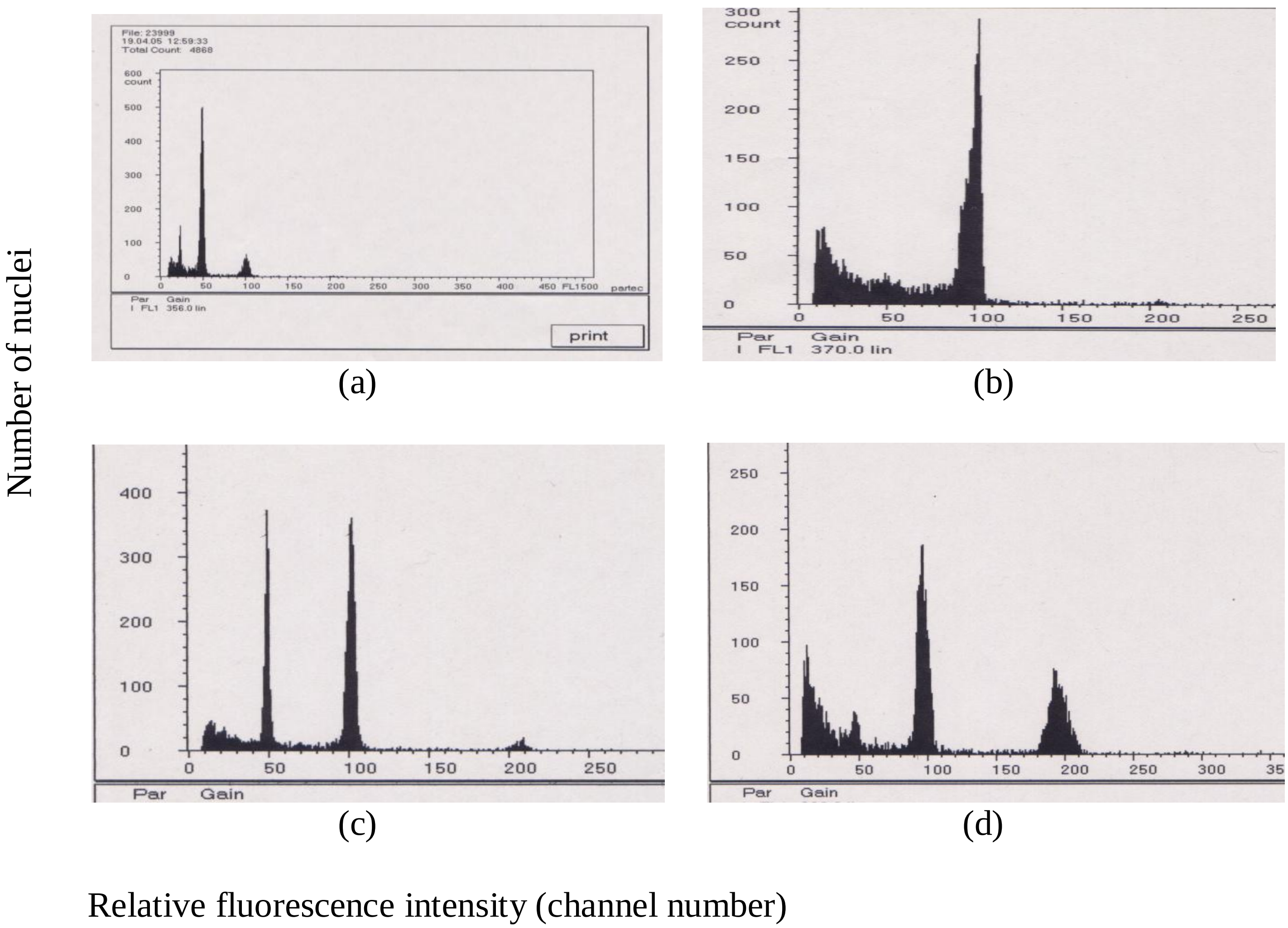

Figure 3. Flow cytometric profiles of 'Egusi' melon plants treated with oryzalin. The peaks of the horizontal axis correspond to relative nuclear DNA content, which is expressed as the fluorescence intensity. The number of nuclei is shown on the vertical axis: (a) DNA content of leaves of control plant (diploid = 2n); DNA content of leaves of treated plants (b) tetraploid = 4n; (c) mixoploid = 2n+4n; (d) mixoploid = 4n+8n

Similar increases in the numbers of pods and branches per plant have also been reported by Biswas and Datta (1988) in *Trigonella foenumfraeum* using 0.25% ethyl methane sulphonate. Moreover, high yielding variants developed through the use of low concentrations of colchicine in various crop species have also been reported (Bragal, 1955). Taken together, the implications of these observations are that bigger pod variants, which could probably be polyploids and produce more seeds, can be derived from 'Egusi' following treatment with low concentrations of either oryzalin or colchicine.

Our flow cytometric studies confirmed that similar to observations made earlier where it was demonstrated that polyploidized plants had larger flowers and bigger fruits in apple (Solov'eva, 1990), most of the antimitotic agent-treated plants as well as the mutants derived from them with bigger pods in the current study were actually polyploids, though some were mixoploids (2n/4n or 4n/8n). These results were expected as they are quite in agreement with the general tendency of polyploids to have larger cells (Levin, 1983), presumably because of the additional amount of DNA in them compared to their diploid progenitors. Perhaps, this may equally serve as one of the reasons responsible for the longer and broader leaves seen here with the 'Egusi' variants, which in itself may be a reflection of the gigas effect, that is, characteristic of polyploid genomes.

It is of great curiosity that though comparative doses of oryzalin and colchicine have been reported to exert similar effects on gerbera (Tosca et al., 1995), their influences appeared different in 'Egusi' as oryzalin treatment commonly appeared more efficient in influencing the agronomic and morphological characteristics evaluated in the current study than those with colchicine. Ordinarily, this result should not be surprising as oryzalin has been considered to be a more reliable compound for mitotic polymerization in kiwifruit and onion (Chalak and Legave, 1996). It is possible that the differences between the observations in gerbera and those reported earlier in kiwifruit and onion as well as those observed in the current study for 'Egusi' may have arisen purely as a result of the fact that the mechanisms of action may be species specific (Van Tuyl et al., 1992). It is equally reasonable to speculate that the higher interference of oryzalin with the Ca^{2+} transport systems operating in the cell organelles, which affects the role of Ca^{2+} in microtubule assembly (Weisenberg, 1972) that have been described to be approximately ten-fold greater than those with colchicine (Okamura, 1980), may have increased its efficacy of action on the morphological traits of the 'Egusi' mutants reported here. It is a common belief that oryzalin and colchicine can both induce chromosome doubling because of their affinity for tubulin, a protein that serves as the building block of microtubules. It is therefore explicable that the action of oryzalin on microtubules could be effected through two mechanisms. First, is its interference with Ca^{2+} transport systems and second, is through the formation of an oryzalin-tubulin complex, which together may have led to more microtubule depolymerization that consequently results in higher rates of polyploidy. Colchicine, on the other hand, only affects microtubule depolymerization through formation of the colchicine-tubulin complex without any interference with Ca^{2+} transport systems. This obviously may have been responsible for the lower effects with colchicine seen here with 'Egusi'.

Collectively, the results reported in this study give credence to the fact that oryzalin may possibly have a higher affinity for plant tubulin than colchicine and might be a better chromosome doubling agent for 'Egusi' melon, especially at low concentrations and at longer treatment durations. With colchicine, the reverse appears to be the case as treatment of the seeds with higher concentrations for longer durations appeared more effective than when treatment was with lower levels; quite in consonance with the results reported by Jaskani et al. (2004), who equally demonstrated that colchicine, seems to be more effective at longer treatment durations. This requirement of oryzalin at lower concentrations (for example, 0.001 and 0.0001 M), which may be attributed to its higher affinity for tubulin heterodimers during assembly of plant microtubules, appears to be an advantage to the plant breeder as it is safer to use because of its lower affinity for animal microtubules (Hansen and Andersen, 1996) and cheaper to purchase compared to colchicine that has carcinogenic effects on humans and it is expensive.

Conclusion

The fact that most of the traits developed newly with the antimitotic agents in the current study were seen to be fixed and showed better performance in the mutants than the untreated control is an indication of the induction of considerable genetic variability, which can serve as an available resource pool. The practical utility of this development is that the yield of 'Egusi' melon can be consistently improved through the production of mutant lines using low concentrations of antimitotic drugs. Such improved lines can be backcrossed with traditional varieties and the products possibly introgressed into conventional breeding programmes for further improvement of this economically important crop.

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