Euphytica **64**: 81–89, 1992.

Heritable tissue culture induced variation in *Zinnia marylandica*

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Received 3 March 1992; accepted 30 September 1992

Key words: interspecific hybrid, somaclonal variation, *Zinnia*

Summary

Adventitious shoots of *Zinnia marylandica,* an amphidiploid with limited genetic segregation, were regenerated from cotyledonary tissue on Murashige-Skoog (MS) media containing 0.2 or 22.2 μM thidiazuron (TDZ) and grown through flowering. Fisher's Test for Equal Variance indicated tissue culture induced plants had more variation than seed-derived control plants. Twelve of 149 (8%) plants derived from 0.2 μM TDZ and three of 23 (13%) plants from 22.2 μM TDZ had variant characters. Aberrant characteristics in self-pollinated variants included plant height, fertility, flower color and morphology, and were sexually transmitted, indicating genetic change had occurred. Aberrant characteristics not observed in regenerated plants arose in progeny.

Abbreviation: TDZ – thidiazuron

Introduction

Traditional methods of breeding for crop improvement are ineffective for *Zinnia marylandica* (Spooner et al., 1991), a disease-resistant *Z. angustifolia* × *Z. elegans* amphiploid (Boyle & Stimart, 1982; Terry-Lewandowski et al., 1984). Fully homologous chromosomes preferentially pair and homoeologous pairing is eliminated, consequently there is limited genetic recombination between the two parental species' genomes. These zinnias may be classified as segmental allopolyploids, having limited to no segregation in subsequent generations (Stebbins, 1950). Self-pollination of zinnia amphiploids produces seed with essentially clonal uniformity. Backcrossing to diploid *Z. elegans* × *Z. angustifolia* or crossing with tetraploid *Z. elegans* 'State Fair' resulted in sterile progeny (T. Boyle, personal communication), making traditional methods of sexual breeding ineffective. New morphological variants which retain amphiploid disease resistance would be valuable for crop improvement.

Somaclonal variation is genetic variation generated during tissue culture (Larkin & Scowcroft, 1981) and is viewed as an option for obtaining genetic variation. Variation has been observed in over thirty species of agronomic and ornamental plants regenerated from *in vitro* culture (Scowcroft, 1985). The high frequency of leaf and flower morphology and color variants in regenerated plants was used to develop *Pelargonium graveolens* 'Velvet Rose' (Skirvin & Janick, 1976a). Evans & Bravo (1986) regenerated adventitious plants from *Nicotiana alata* 'Nicki Red' and observed variations in flower and leaf shape, plant height and pollen viability. Increasingly disorganized growth of cultured tissue may allow for more genetic rearrange-

ment and variation in regenerated plants (Reisch, 1983). Somaclonal variants of many plants including *Avena sativa, Lactuca sativa, Nicotiana tabacum,* and *Triticum aestivum* have transmitted variation to progeny through sexual reproduction (Scowcroft, 1985). *In vitro* culture may induce chromosomal changes enhancing gene recombination in interspecific hybrids (Orton, 1980). Lentini et al. (1990) recovered insect-resistant plants from interspecific potato hybrids grown *in vitro*.

Research results (Stieve, 1991) demonstrated adventitious shoots formed on cultured *Z. marylandica* embryo tissue. Optimum conditions for adventitious shoot formation were 16 day old cotyledons oriented adaxial surface down on MS salts and organics supplemented with 0.2 μM TDZ, based on number of adventitious shoots formed and percent embryos forming adventitious shoots. Embryos cultured on 22.2 μM TDZ produced more callus and took longer to form adventitious shoots.

The purpose of this research was to evaluate variation of adventitious-derived plants from media containing two levels of TDZ, and determine if variation was heritable.

Materials and methods

Study 1: Variation of adventitious Zinnia marylandica *plants*

A seed-derived orange flowered *Zinnia angustifolia* × *Z. elegans* 'Thumbelina Mini-Salmon' amphiploid (*Z. marylandica,* Spooner et al., 1991) was propagated asexually and maintained in University of Wisconsin-Madison glasshouses as described previously (Stieve, 1991).

In August and December, 1989, flower heads on clonal plants were self-pollinated. Two seeds were removed randomly from each flower head 16 days after pollination, surface disinfested for 20 min in 1.6% sodium hypochlorite (30% Clorox) with one drop Tween 20 per 100 ml, rinsed in sterilized water three to four times, and held under sterile conditions in water in a laminar air flow cabinet. Embryos were excised from seed coats under a dissecting microscope, slit three to four times on adaxial surfaces and cultured adaxial surface in contact with medium.

Culture media consisted of MS medium salts and organics (Murashige & Skoog, 1962; Stieve, 1991) supplemented with 0.2 or 22.2 μM TDZ (N-phenyl-N′-1,2,3-thidiazol-5-ylurea). Media was brought to volume and adjusted to pH 5.6. Magenta GA-7 vessels containing 75 ml media were used. Containers and media were autoclaved 20 min at 120° C and 1.06 kg cm^{-2}.

In August, 16 cotyledons were placed randomly onto 22.2 μM TDZ, only one cotyledon of each embryo was used. In December, eighty cotyledons were isolated and four cotyledons from two embryos of the same flower were cultured in one vessel on 0.2 μM TDZ. Cultures were maintained at 28° C under continuous cool-white fluorescent light (22 $\mu M^{-2} s^{-1}$). After 21 days of culture cotyledons were subcultured onto MS medium salts and organics supplemented with 4.4 μM benzylaminopurine (BAP) for adventitious shoot elongation. Cultures were grown for two weeks at which time adventitious shoots forming on cotyledons on 0.2 μM TDZ were separated and transferred to 1/2 strength MS medium salts for rooting. Cotyledons started on 22.2 μM TDZ formed adventitious shoots after subculturing for 4 weeks on media containing BAP. Adventitious shoots were separated and rooted as above. Roots became visible in two to three weeks, plantlets (R_0 generation) were planted in 4 cm square cell packs in a 50:50 (v:v) sphagnum peat:perlite medium, acclimated in fog for three days, intermittent mist for three days, and placed in the glasshouse. The two groups of adventitious plants were placed into the glasshouse at approximately the same time. Control plants of the amphiploid parent were grown from seed at this time. Seeds were sown in the same medium used for adventitious plants and placed in fog until cotyledons emerged.

Adventitious-derived and control plants were transplanted to 10 cm square pots containing a pasteurized medium of soil:sand:sphagnum peat (v:v:v) when two pairs of true leaves emerged. Plants were grown under glasshouse conditions

previously outlined and fertilized every third day with 20N-8.7P-16.6K fertilizer at 200 ppm nitrogen.

One hundred forty-nine of 510 adventitious shoots and 23 of 31 adventitious shoots formed on 0.2 and 22.2 μM TDZ, respectively, rooted and flowered. There were 86 control plants grown to flowering. Data were taken when the first three flowers on a plant had two rows of disc florets expanded. Parameters measured included leaf length and width of one leaf of the first pair of opposite leaves below the flower, internode length below the first pair of opposite leaves, peduncle length, flower head diameter measured from tips of opposite ray petals, ray petal length and width, number of ray petals per flower head, and percent seed set. Three measurements of each parameter were made per plant.

Seed set data were collected by self-pollinating flower heads when two rows of disc florets expanded. Pollen was transferred from disc florets to stigmas of ray and disc florets with a fine hair brush every other day over a six day period, between 1200 and 1400 hr. Flower heads were matured on the plant and collected three weeks after pollination. Seeds were black and dark brown in color when harvested and those which remained plump when pressure was applied with a scalpel were considered viable. Percent seed set was calculated by dividing the number of plump seeds by the total number of seeds and multiplying by 100. Values for percent seed set were rounded to the nearest whole percent.

Fisher's Test for Equal Variance (Snedecor & Cochran, 1980) was performed on data. Percent seed set data were normalized by arcsine transformation of the square root of percent seed set divided by 100.

Study 2: Heritability of variation

Control plants and R_0 plants with variant characteristics were self-pollinated as described previously, and seed harvested in spring 1989.Seeds were surface disinfested for 20 min in 1.6% sodium hypochlorite (30% Clorox) with one drop Tween 20 per 100 ml, rinsed in sterilized water three to four times, and sown on moistened filter paper in Petri dishes. Control and R_1 (selfed R_0) seedlings were transplanted into 4 cm square cell packs, grown until two pairs of true leaves emerged, transplanted into 10 cm square pots and grown in glasshouse conditions through flowering.

Data were taken on the variant characteristic for which the parent was selected. Fasciated flower heads and ray petal spots were defined as present if observed on at least one flower. Upwardly curved ray petals were defined as present when observed on at least half the flowers of a plant.

Fisher's test on control generations showed similar variation for petal length, flower diameter and percent seed set. Therefore, environmental conditions were considered similar between Studies 1 and 2, and R_1 progeny were compared to R_0 parents.

Results

Study 1

Leaf length and width, internode length, ray petal number, and seed set were on average largest in control plants (Table 1) when compared to TDZ-derived plants. Control plant means for peduncle length and petal width were less than TDZ-derived plants. Flower diameter and petal length control means were similar to 0.2 μM TDZ derived plants, but larger than 22.2 μM TDZ means. On average 0.2 μM TDZ means were largest for leaf length, flower diameter, petal length, and seed set when compared to 22.2 μM TDZ values. Mean values for leaf width, internode length, petal width, and ray petal number were similar between TDZ treatments. Peduncle length was largest on 22.2 μM TDZ.

Variances of R_0 plants derived from 0.2 μM TDZ were significantly greater than control plants for leaf length and width, peduncle length, flower diameter, and ray petal length, width and number (Table 1). Internode length and seed set variances

were similar to control plants. Plants derived from 22.2 μM TDZ had variances significantly greater than control plants for internode and peduncle length, petal number, and seed set, and greater variance than 0.2 μM TDZ-derived plants for four of nine parameters observed.

Study 2

Twelve variant R_0 plants were identified from 0.2 μM TDZ (8.0% of the population) and three from 22.2 μM TDZ (13.0% of the population). Aberrant characteristics identified in 0.2 μM TDZ-derived plants included tallness (line 3-1-2); dwarfness (lines 2-1-10, 5-1-5 and 5-1-6); increased seed set (line 17-2-4 and 17-2-6); fasciated flower heads (line 7-1-2) (Fig. 1), upwardly curved ray petals (line 5-1-7) (Fig. 2), striped ray petals (line 16-2-2) (Fig. 2); and darker red-orange (line 17-2-5) (Fig. 1) or muted orange ray petals (line 16-2-3). Two flowers with muted orange ray petal color had pink areas on petal margins and yielded pink-flowered progeny when selfed. Additionally, two regenerated plants had variegated leaf sectors. These areas were confined to plant sectors not near flower stems. Variegated areas became less distinct as plants aged and normal flowers were produced, so variegation was not studied further.

Aberrant characteristics identified in 22.2 μM TDZ-derived plants included large disc diameter of flower heads with short ray petals (line BxT3-1) (Fig. 2) and green spots on ray petals (lines BxT3-14 and BxT3-15) (Fig. 1). Two plants producing green ray petal spots had non-spotted flowers until approximately five flowers opened, after which 75% had spotted rays.

Three examples were observed where the same variant arose in cotyledons of one embryo: dwarfness in lines 5-1-6 and 5-1-7, high seed set in lines 17-2-4 and 17-2-6, and green spots on ray petals in lines BxT3-14 and BxT3-15. Two seedlings from

Table 1. Summary of *Zinnia marylandica* characteristics in seed propagated (control) or adventitious shoots (R_0) derived from 0.2 or 22.2μM thidiazuron

Population/analysis	Size (mm)								
	Leaf		Internode length	Peduncle length	Flower diameter	Ray petal			Seed set (%)
	length	width				length	width	number	
Control[Z]									
Mean	53.1	24.4	65.2	42.2	48.8	18.0	10.4	14.5	26.3
Range	36.7–69.0	15.7–33.2	38.3–99.7	19.3–79.0	34.3–57.0	12.2–21.3	8.2–12.5	12.5–18.7	0.0–71.7
Variance	56.1	14.8	140.5	154.7	17.9	2.8	0.8	1.6	0.1[Y]
R_0 0.2 μM TDZ[X]									
Mean	49.4	20.4	46.6	48.9	48.7	18.5	11.5	12.2	17.9
Range	25.0–72.5	10.2–35.7	14.0–82.3	14.0–99.7	32.3–60.7	11.3–23.3	6.3–16.7	7.7–21.0	0.0–80.0
Variance	90.9	24.0	124.5	281.9	33.2	5.6	2.8	6.2	0.1[W]
Fisher's Test[V]									
versus control	**	**	NS	**	**	**	**	**	NS
R_0 22.2 μM TDZ[U]									
Mean	46.2	21.0	48.9	61.1	46.7	17.3	11.4	12.1	1.1
Range	37.0–72.5	16.0–28.2	31.0–111.0	20.7–110.0	39.7–54.3	13.3–21.2	10.0–12.7	8.0–16.7	0.0–6.6
Variance	64.2	10.8	377.0	361.2	13.8	2.9	0.6	6.3	3.4[T]
Fisher's Test									
versus control	NS	NS	**	*	NS	NS	NS	**	**
versus 0.2 μM TDZ	NS	NS	**	NS	NS	NS	NS	NS	**

[Z, X, U] Based on 86, 141 and 23 plants, respectively.
[Y, W, T] Based on 84, 138 and 22 plants, respectively.
[V] Comparison by Fisher's Test for Equal Variance.

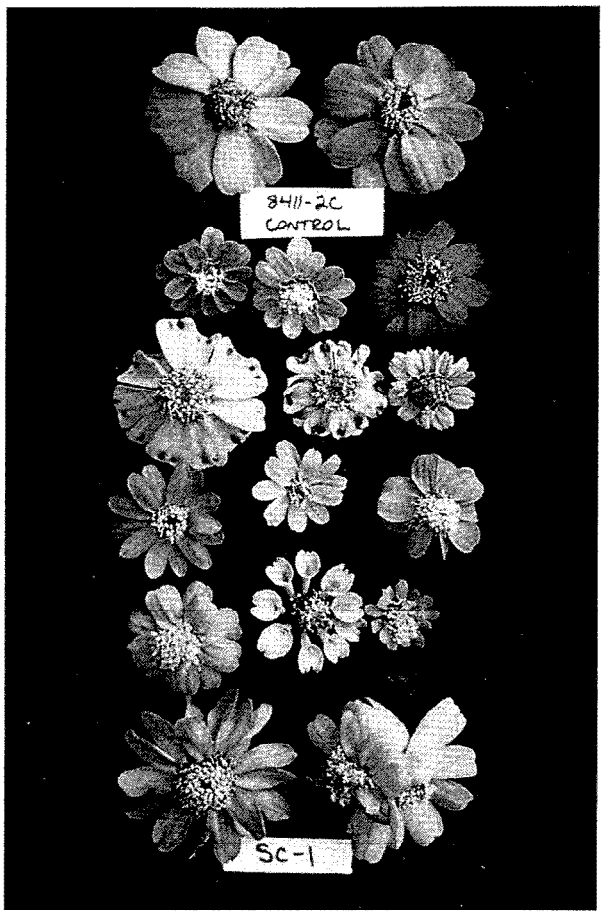

Fig. 1. Zinnia marylandica 8411-2c seed derived control plants (row 1) and R_1 adventitious derived progeny showing line 17-2-5 dark red-orange rays (row 2, right), line BxT3-14 and BxT3-15 green spotted rays (row 3; left, center) and short dentate rays (row 3, right), line 17-2-5 crested disc florets (row 5, left), line 16-1-4 tubular ray florets (row 5, center), and line 7-1-2 fasciated flower heads (row 6, right).

lines 14-1-4 (unreported) and 7-1-2 produced adventitious shoots on cotyledons. Shoot apical meristems of these seedlings never developed and plants were lost.

Tallness was transmitted sexually to line 3-1-2 progeny and dwarfness to line 2-1-10, but not 5-1-5 or 5-1-6 (Table 2). Tallness and dwarfness were due to increased or decreased internode and peduncle

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Fig. 2. Upwardly curved ray petals of *Zinnia marylandica* line 5-1-7 (a) and striped ray petals of line 16-2-2 (b) adventitiously derived from 0.2 μM thidiazuron; large disc area and short ray petals of line BxT3-1 adventitiously derived from 22.2 μM thidiazuron (c).

Table 2. Mean and range of *Zinnia marylandica* characteristics from tissue culture-derived adventitious plants (R_0) and self-pollinated progeny (R_1)

Trait	Line	Generation	No. plants observed	Internode length (mm)	Peduncle length (mm)	Plant height (cm)	Flower diameter (mm)	Disc diameter (mm)	Petal length (mm)	Seed set (%)
	Control	Unknown	45	58.8 ± 2.5[z] 28.7–93.0	42.4 ± 2.1 18.7–74.3	55.0 ± 1.8 31.0–69.0	47.1 ± 0.58 36.0–55.5	12.5 ± 0.03 9.0–15.5	18.1 ± 0.30 13.3–23.0	17.5 ± 3.8 0.0–39.6
Tallness	3-1-2	R_0	1	75.0 ± 6.9 63.0–87.0	88.7 ± 11.3 67.0–105.0					
		R^1	111	93.7 ± 2.4 33.3–152.7	83.0 ± 3.8 10.0–181.7	60.4 ± 1.1 35.0–84.5				
Dwarfness	2-1-10	R^0	1	41.2 ± 4.2 34.0–48.5	29.7 ± 3.0 24.0–31.0					
		R_1	10	34.8 ± 2.6 21.7–48.0	22.0 ± 1.8 13.7–29.3	32.6 ± 2.6 22.0–43.5				
Dwarfness	5-1-5	R_0	1	45.0 ± 8.0 37.0–61.0						
		R_1	14	74.7 ± 2.2 59.7–88.3		54.9 ± 1.0 49.5–58.5				
Dwarfness, high seed set	5-1-6	R_0	1	36.3 ± 1.8 34.0–40.0						65.3 ± 2.2 60.9–69.1
		R_1	14	64.3 ± 3.3 45.3–94.0		50.8 ± 1.8 39.0–63.0				41.7 ± 4.6 16.0–55.8
High seed set	17-2-4	R_0	1							48.4 ± 5.7 35.0–58.0
		R_1	9							39.2 ± 2.5 27.2–52.9
High seed set	17-2-6	R^0	1							65.3 ± 1.9 61.7–68.3
		R_1	9							42.2 ± 3.3 29.4–64.6
Disc area diameter and petal length	BxT3-1	R_0	1				39.7 ± 2.4 35.0–59.3	15.9 ± 1.5 11.0–19.5	13.3 ± 1.0 11.5–15.0	
		R_1	16				42.6 ± 1.4 35.5–59.3	17.0 ± 0.3 15.5–20.0	15.6 ± 0.4 13.0–19.3	

[z]Numerator represents mean and standard error, respectively; denominator represents range.

lengths. Increased seed set was transmitted sexually in lines 5-1-6, 17-2-4 and 17-2-6. R_1 plants had lower seed set than R_0 plants, but exceeded control seed set by about 2.3 fold (Table 2). Disc area diameter and short petal length were transmitted sexually to progeny of line BxT3-1. R_1 ranges for internode length, peduncle length, plant height, disc diameter, petal length, and seed set were larger than R_0 plants, reflecting the larger number of observations going into them (Table 2). Flower diameter ranges of R_0 and R_1 generations were similar.

Fasciated flower heads were transmitted sexually and observed in 40% of line 7-1-2 (Table 3). Upwardly curved ray petals were transmitted to all progeny of line 5-1-7. Distorted ray petals of line 16-1-4 were transmitted to 33% of progeny. Ray petals of one plant were fused and tubular near the flower head and flared at the tip (Fig. 1). Ray petal striping was not inherited by progeny of line 16-2-2 (Table 3), but appeared to segregate into solid colored lighter orange or darker red-orange ray petals. Darker red-orange rays of line 17-2-5 were not inherited by progeny (Table 3), but a new aberration of crested disc florets was observed in one plant (Fig. 1). All progeny except crested had upward curved ray petals. Muted orange rays of line 16-2-3 were transmitted sexually to 81% of R_1 plants (Table 3). Additionally, 2% displayed darker red-orange ray petals and were tall, 10% lighter yellow-orange ray petals, 2% solid orange ray petals, 50% of which had two green spots near the tip, and 4% had pink rays and were short. One pink-flowered plant quit flowering after one month and new growth ceased. Green ray petal spots were inherited by two of seven progeny for BxT3-14 and none of the six progeny observed in line BxT3-15 (Table 3). A new aberrant flower type with very short dentate ray petals arose in progeny of both lines (Fig. 1). All progeny of these two lines grew slowly and took up to three months to flower.

Discussion

Research on *Zinnia marylandica* showed regeneration of plants from tissue culture results in many variations in plant height; fertility; and flower color and morphology. Variant characteristics included tallness; dwarfness; high seed set; large disc area diameter; fasciated flower heads; and ray petal curvature, distortion, striping, spotting, and color

Table 3. Heritability of *Zinnia marylandica* characteristics from tissue culture-derived adventitious plants (R_0) to self-pollinated progeny (R_1)

R_0[Y] trait observed	Line	R_1 generation[Z]		
		Number observed	Trait observed	% with character
Flower head fasciation	7-1-2	15	Flower head fasciation	40
Upward ray petal curvature	5-1-7	22	Upward ray petal curvature	100
Distorted rays	16-1-4	6	Distorted rays	33
Striped rays	16-2-2	10	Striped rays	0
Dark red-orange rays	17-2-5	50	Dark red-orange rays	0
			Upward ray petal curvature	100
			Crested discs	2
Muted orange rays	16-2-3	48	Muted orange rays	81
			Dark red-orange rays	2
			Light yellow-orange rays	10
			Pink rays	4
			Green ray petal spots	2
Green ray petal spots	BxT3-14	7	Green ray petal spots	29
Green ray petal spots	BxT3-15	6	Green ray petal spots	0

[Z] Self-pollinated progeny of tissue culture-derived plants.

[Y] Plants regenerated from tissue culture by adventitious shoots.

variations. All variants except striped ray petals and dark red-orange ray petal color were transmitted sexually to some degree from R_0 to R_1 plants, indicating that genetic changes rather than epigenetic changes had occurred. New aberrant characteristics of pink ray petals, crested disc florets, green spots on ray petals, upwardly curved ray petals, and tubular ray petals, not observed in the R_0 generation, arose in subsequent progeny. This suggests that recessive genetic changes occurred in culture, genetic change occurred in R_0 plants, or that R_0 genomes are unstable and genetic rearrangement continues out of culture. The appearance of new variants in self-pollinated progeny of regenerated plants may demonstrate the need to study future generations of phenotypically normal R_0 plants to detect segregating characteristics. Seed-derived control plants displayed normal and uniform growth.

Fisher's Test for Equal Variance showed 0.2 μM TDZ-derived plants had more variation than control or 22.2 μ-derived plants for most characteristics observed. This suggests that higher TDZ levels do not induce more genetic variation, therefore 0.2 μM TDZ is adequate for obtaining somaclonal variants of *Z. marylandica*.

Adventitious shoots may be formed by one or a few vegetative daughter cells of one original cell (Broertjes & Keen, 1980), which may explain how plants having the same variant characteristics of high seed set, green ray petal spots and epigenetic dwarfness arose. One cotyledon cell may have mutated early in culture and formed many mutant daughter cells, which could then have developed into distinct adventitious shoots having the same mutation. Alternatively, a pre-existing mutation may have been present in cotyledon tissue from which one or more adventitious shoots formed, indicating somatic instability of the *Z. marylandica* embryo genome may have been responsible for observed variations.

Variation in tissue culture may in part be due to the tissue culture environment allowing for increased genetic recombination (Skirvin & Janick, 1976b) through homoeologous pairing, allowing pre-existing genetic differences of *Z. elegans* and *Z. angustifolia* parental species to be expressed. Many cytogenetic changes have been observed in somaclonal variants including single gene mutations (Evans & Sharp, 1983) and altered chromosome numbers and structures (D'Amato, 1977). Mitotic crossing over and transposable element activation may account also for some of the variation detected in regenerated plants. Karyotypic analysis of *Z. marylandica* regenerated plants would be useful in detecting cytogenetic changes in variant plants, but high chromosome number ($2n = 46$) and small size make this difficult. Based on the fact that more variation was present in 0.2 μM TDZ-derived plants, reduced fertility of 22.2 μM TDZ-derived plants may be epigenetic rather than cytogenetic, reflecting the longer time taken to regenerate *in vitro*.

While the phenomenon of somaclonal variation has been documented widely its underlying mechanism is not understood. Lee & Phillips (1988) have proposed a mechanism for somaclonal variation based on late replication of heterochromatin. Since heterochromatic regions replicate after euchromatic regions (Lima-De-Faria, 1969) their integrity may be particularly vulnerable to any fluctuations occurring in a cultured cell's cycle. The association between *in vivo* chromosome breakage and sites of late-replicating heterochromatin has been documented for *Avena sativa* (McCoy et al., 1982) and Crepis capillaris (Sacristan, 1971). Lee & Phillips suggest transposable elements may be activated as early as the first division following heterochromatin breakage, representing the plant genome's response to stress, as suggested by McClintock (1983, 1984). Ray petal striping observed on one regenerated *Z. marylandica* may be an example of this, the link between transposable elements and flower pigmentation patterns has been studied in *Antirrhinum majus* (Fincham, 1987) and *Petunia* sp. (Wijsman, 1986). Groose & Bingham (1986) regenerated *Medicago sativa* from callus and identified a white-flowered mutant which frequently reverted when recultured, most simply explained by activation of a transposable element.

Zinnia marylandica was selected for study because it was resistant to three major zinnia pathogens but displayed no morphological variation. Somaclonal variants of commercial interest, including

high seed set and novel flower pigmentation patterns and shapes, were obtained and should be tested for continued disease resistance, since it may have been lost or mutated as a result of genetic change in culture. Future research with variant plants may be hindered by reduced fertility of variant plants, since many appear to set little or no seed. One solution to this may be crossing morphological variants with high seed setting variants, which may promote increased seed set. Placing mutations of interest into the amphiploid genotype by backcrossing may prove useful in the development of commercially viable cultivars. Also of interest is putting variant plants through another tissue culture regeneration cycle to see if they are genetically unstable, perhaps reverting to the parental phenotype or to an even more aberrant phenotype.

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