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EFFICACY OF ORYZALIN AS A POTENT CHEMICAL FOR INDUCTION OF *IN VITRO* POLYPLOIDY IN ASIATIC HYBRID LILY (VAR. POLLIYANNA)

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ABSTRACT

The efficacy of a mitotic inhibiting compound, oryzalin was tested on *in vitro* polyploidy induction in Asiatic hybrid lily var. Pollyanna. Bulb scale segments were exposed to different concentrations of oryzalin (0, 0.001, 0.003, 0.005, 0.007 and 0.01%) for 2, 4 and 6 hrs. Concentration and time duration interaction is not significantly different for explant survival, bud sprouting, number of shoots per explant and leaf length. But main effect of concentration and time duration shows significant different. Increase in the concentration of oryzalin resulted in reduced explant survival and delayed bud sprouting. Further it revealed that few shoots per explant with stunted growth were observed at initial stages. However, delayed rooting with slow growth was observed in all the treatments after transferring those sprouted explants to the rooting medium. The root tips of treated plants were examined for ploidy determination. The highest percentage of tetraploids (45) and the highest efficiency of tetraploid conversion (1.1) were recorded with the exposure to 0.001% oryzalin for 6 hrs. In higher oryzalin concentrations (>0.001%), diploids and mixoploid/aneuploid plants were regenerated, while at the lowest oryzalin concentration (0.001%), either diploids or tetraploids were observed.

KEYWORDS: Bulb scales, Explant, *In vitro* polyploidy, *Lilium*, Oryzalin

INTRODUCTION

Lilium (*Lilium* sp.) is one of the most fascinating bulbous flower crops used as a cut flower and potted plant all over the world. Natural species of Asiatic lilies are mostly diploid (2n = 24), but some triploid forms (2n = 3x = 36) exist, which are sterile. Tetraploid forms have proved to be more robust in their growth habit having larger flowers, deeper and brighter coloured petals, stronger and sturdy stems, thicker texture of tissues and higher resistance to pests and diseases (Levin, 1983). At present, many of the efforts are directed towards the creation of polyploid cultivars in lilies.

Chromosome doubling has been widely used in plant breeding programmes to create new cultivars (Griesbach, 1990) and for the restoration of inter-specific or inter-generic hybrids (Anderson *et al.*, 1991). Some interference in the meiosis can result in the spontaneous formation of polyploids in lilies. The production of tetraploid plants has been accomplished artificially by treating apical meristems, seedlings, seeds or bulb scales of

diploid plants with chemicals like colchicine. However, colchicine has a toxic effect, thus decreasing the survival rate and regeneration in treated cultures and in some cases shows undesirable mutagenetic changes in the plants (Van Tuyl *et al.*, 1992). Oryzalin is a herbicide, which inhibits mitotic activity by binding to plant tubulins and leads to high micro-tubule de-polymerizing activity. Moreover, oryzalin is characterized by its relative low toxicity than the other chemicals such as trifluralin, caffeine, amiprophos-methyl, pronamide and N_2O gas used for chromosome doubling in many crops (Eeckhaut *et al.*, 2006; Van Tuyl *et al.*, 1992).

A high frequency of chimeras is normally associated with chromosome doubling treatments *in vivo*. It has proved that chromosome doubling *in vitro* is an excellent technique due to its high efficiency of polyploid production and few chimeras (Chen and Goeden-Kallemeyn, 1979). Hence, there is a great scope for using *in vitro* techniques in polyploidy studies in *Lilium* and other ornamentals.

This paper describes a simple method of using oryzalin for induction of *in vitro* polyploidy in an Asiatic hybrid lily var. Pollyanna and also its multiplication.

MATERIALS AND METHODS

Plant material and pre-treatments of bulb scale

Healthy bulb scales of Asiatic hybrid lily var. Pollyanna were separated from mature bulbs stored at 4°C in a moist coco-peat for four months. These bulb scales were rinsed with running tap water for 20-30 min. until all attached soil particles were removed. Explants were put into a conical flask and agitated with a solution containing carbendazim (0.2 %), 8 – hydroxy quinnoline citrate (200 ppm), GA_3 (100 ppm) treatment combinations for 2 hrs, followed by three rinses with sterile distilled water.

Oryzalin treatment

The pre-treated bulb scales were transferred to the lamina air-flow hood. Scales were dissected to longitudinal segments of 4 mm width. These segments were then treated with five oryzalin concentrations for 2, 4 and 6 hrs. Oryzalin (PESTANAL®) as procured from Sigma-Aldrich Chemicals Private Limited, St. Louis, USA. Twenty mg of oryzalin was dissolved in 1 ml of Dimethyl sulfoxide (DMSO) and then the stock solution was diluted with sterile distilled water to prepare the working solutions (0, 0.001, 0.003, 0.005, 0.007 and 0.01 %, w/v). Enough solution was added to cover the entire scale segments. After soaking in different chemical solutions, the explant was rinsed three times with sterile distilled water.

Surface-sterilization of explants, inoculation and incubation conditions

Bulb scales were agitated in 0.1 % $HgCl_2$ for 7 min, followed by three rinses with sterile distilled water before inoculation. The chemical-treated explants were cultured on a full-strength MS basal medium (Murashige and Skoog, 1962) supplemented with 3 % sucrose and 0.8 % agar (w/v) along with 0.2 mgl^{-1} NAA and 1.0 mgl^{-1} BAP. One scale segment was placed per test tube with the abaxial side touching the media surface.

Cultures were incubated at 24±1°C under white fluorescent light of 1.5 K Lux and a light/dark cycle of 16/8 hrs. All cultures were sub-cultured on fresh medium every four week interval.

***In vitro* rooting of shoots**

The bulblets obtained from the bulb scale segments were subsequently transferred onto a half-strength MS medium containing 1.0 mgl^{-1} IBA. After four days of sub-culturing, actively growing roots of 1 cm in length, were excised for ploidy determination.

Cytological studies

The just initiated roots of 1 cm long with rapidly growing root tips were taken from the Polyanna bulbs and the newly sub-cultured plantlets for cytological study. Chromosomes were counted using the standard acetocarmine technique (McClintock, 1929).

Statistical analysis

All experiments were carried out in a complete randomized design (CRD) with a factorial arrangement of treatments where necessary, with two replicates per treatment. Each treatment had at least 15 test tubes/conical flasks. Data were subjected to analysis of variance (ANOVA) (SAS Institute, 2002). Means were analyzed using method Tukey (MSD) at $p<0.05$. Percentage data were transformed using arc sin transformation before analysis.

RESULTS AND DISCUSSION

Initial cytological study

Cytological study on root tips taken from Polyanna bulbs revealed that the diploid chromosome number was $2n = 2x = 24$ (Figure 1).

Pre culture treatment of oryzalin on culture establishment and shoot regeneration

Analysis of data with General Linear Model (GLM) for the CRD design with factorial structure, revealed that the interaction between the concentration of oryzalin and time of exposure was not significant ($p>0.05$) for explant survival, bud sprouting, duration for sprouting, number of shoots per explant and leaf length. Therefore, the main factors, oryzalin concentration and time of exposure are separately discussed and the results, accordingly, are given in Table 1 and 2.

Table 1. Effect of pre-culture treatment of oryzalin on culture establishment and shoot regeneration in bulb scale segments of Asiatic hybrid lily var. Pollyanna.

Concentration (%)	*Explant survival (%)*	*Bud sprouting (%)*	*Duration for sprouting (days)*	*No. of shoots per explant*	*Av. length of leaves (cm)*
0 (Control)	92.22 ^{a}	91.11 ^{a}	15.50 ^{c}	5.50 ^{a}	7.63 ^{a}
0.001	62.22 ^{b}	57.78 ^{b}	17.17 ^{bc}	2.67 ^{b}	4.67 ^{b}
0.003	40.00 ^{c}	35.55 ^{c}	19.17 ^{b}	2.83 ^{b}	4.50 ^{b}
0.005	24.44 ^{d}	20.00 ^{d}	19.50 ^{ab}	2.33 ^{b}	3.83 ^{bc}
0.007	27.78 ^{de}	22.22 ^{d}	19.67 ^{ab}	2.67 ^{b}	3.12 ^{c}
0.01	15.55 ^{e}	7.78 ^{e}	21.83 ^{a}	2.17 ^{b}	3.03 ^{c}
*MSD	10.78	7.35	2.57	1.33	0..87

Means with the same letters are not significantly different at $p=0.05$. *MSD = Minimum Significant Difference

Among the treated bulb scales, the highest survival (62.22 %) and bud sprouting (57.78 %) were observed with the lowest concentration (0.001 %) of oryzalin. However, higher concentrations have decreased the explant survival and bud sprouting. Similar results of a sharp decrease in *in vitro* plant regeneration with increasing concentration of oryzalin were observed by Tao *et al.* (2003) in ornamental *Alocasia*. Eeckhaut *et al.* (2006) reported high mortality in *Rhododendron* species treated with oryzalin at higher concentrations (>0.01 %) regardless of duration of treatment. However, in contrast, even with the longest soaking period (8 hrs) and the highest concentration (0.01 %) oryzalin, the *in vitro* plant regeneration of tulip has not been affected (Chauvin *et al.*, 2005).

There was no significant difference among the lower concentrations of oryzalin and the control for sprouting date. But sprouting was delayed in highest oryzalin treatments when compared to the control. Significant differences were also found for the average shoots per explant between control and chemical treatments. When the bulb scales were exposed to chemical treatments, the multiplication rate diminished significantly. The treated explants, regardless of exposure time, damage to bulb scale producing less shoots.

The average length of leaves per shoot was also highly significant among the treatments and the control. Significantly higher number of shorter leaves in rosette like appearance was observed in initial growth stage of the treated plants. Similarly, Escandon *et al.* (2006) found that comparatively slow growth of regenerated plants from ploidy inducing treatments of *Bacopa monnieri* under *in vitro* conditions. Shao *et al.* (2003) observed shorter narrow leaves in *in vitro* regenerated ploidy inducing treated pomegranate plants later confirmed as mixoploids. Polyploid plants often display thickened leaves, stunted or slowed growth (Blakeslee and Avery, 1937). The initially retarded and distorted growth of induced autopolyploids has also been reported by Cohen and Yao (1996). According to Chauvin *et al.* (2005) the distortion of growth is usually the result of a mixture of doubled and undoubled chromosomes: and the growth returns to normal when a balance is achieved. According to Levin's (1983) review, tetraploids grow more slowly than diploids throughout their lifespan.

Table 2. Effect of time duration exposure to oryzalin on culture establishment and shoot regeneration in bulb scale segments of Asiatic hybrid lily var. Pollyanna.

Hours of exposure to the chemical	*Explant survival (%)*	*Bud sprouting (%)*	*Duration for sprouting (days)*	*No. of shoots per explant*	*Av. length of leaves (cm)*
2	48.33 [a]	42.22 [a]	19.50 [a]	3.41 [a]	4.86 [a]
4	41.17 [b]	40.00 [a]	18.75 [a]	2.92 [a]	4.29 [b]
6	41.11 [b]	35.00 [b]	18.17 [a]	2.75 [a]	4.24 [b]
*MSD	6.13	4.17	1.46	0.76	0..49

Means with the same letters are not significantly different at p=0.05. *MSD = Minimum Significant Difference

Table 2 shows the effect of different period of exposure (2 hrs, 4 hrs, and 6 hrs) to oryzalin on explant survival, bud sprouting, duration for sprouting, number of shoots per explant and leaf length. The results revealed that longer exposure of the chemical suppressed the explant survival, bud sprouting and leaf length significantly while no effect for duration of sprouting and shoots per explant.

Root induction

Data on the percentage of rooting, duration for rooting (days), number of roots per shoot and the root length (cm) were analyzed following General Linear Model (GLM) procedure for the CRD design with factorial structure. The combination of concentration of oryzalin and time level interaction was not significant ($p>0.05$). Therefore main factors are separately discussed.

The percentage of rooting was not significantly different among the oryzalin treatments and the control (Table 3). However, significantly delayed rooting was observed with chemical treated plantlets as compared to the

control. The number of primary roots per shoot was also significantly different from the control while among treatments was not showed clear significance. The average length of roots was significantly deceased with increasing concentration. At the highest concentration of oryzalin, adverse effect of chemical was evident that delayed in rooting coupled with short roots as compared to control. These results are similar to those of Stebbins (1950) who found that initial growth inhibition with ploidy inducing chemicals was not solely and directly due to treatment effect on mitosis but due to associated physiological changes.

Table 3. Effect of pre-culture treatment of oryzalin on root induction in Asiatic hybrid lily var. Pollyanna.

Concentration (%)	*Rooting (%)*	*Duration for rooting (days)*	*No. of roots per shoot*	*Av. root length (cm)*
0.00	93.33 a	14.17 d	8.67 c	4.28 a
0.001	90.70 a	21.83 c	10.83 a	1.48 b
0.003	83.75 a	22.50 c	10.33 ab	1.07 bc
0.005	81.53 a	23.83 b	9.83 abc	0.90 c
0.007	79.07 a	24.50 b	9.17 bc	0.78 c
0.01	75.00 a	28.33 a	9.50 abc	0.23 d
*MSD	19.83	1.33	1.62	0.45

Means followed by the same letters are not significantly different at p=0.05. *MSD - Minimum Significant Difference

Pre-culture treatment of oryzalin on mitotic chromosomal polyploidization

All the regenerated plants from the oryzalin treatments and some of control plants (2 months after inoculation) were tested for their ploidy and the results are shown in Table 4.

The highest percentage (45 %) of tetraploids (Figure 2; 4x = 48) were observed from the lowest concentration of oryzalin 0.001 % for 6 hrs. There were no mixoploids or aneuploids induced with 0.001 % oryzalin. The present findings on oryzalin treatment as a ploidy inducer lend support from several workers. Van Tuyl *et al.* (1992) proposed concentrations of oryzalin below 0.005 % as effective concentrations for *in vitro* chromosome doubling in lily. Van Tuyl *et al.* (1992) also observed higher frequency of polyploid formation in 0.003 and 0.005 % oryzalin. Similar results were obtained by Vainola (2000) in *Rhododendron*. Tao *et al.* (2003) who reported that treatment with 0.01 % oryzalin for 24 hrs was the most effective concentration for inducing tetraploids in ornamental *Alocasia*. In this study, no tetraploid chromosome number was observed, at the highest concentration of oryzalin of 0.01 %, but only diploids and mixoploids/aneuploids were noticed.

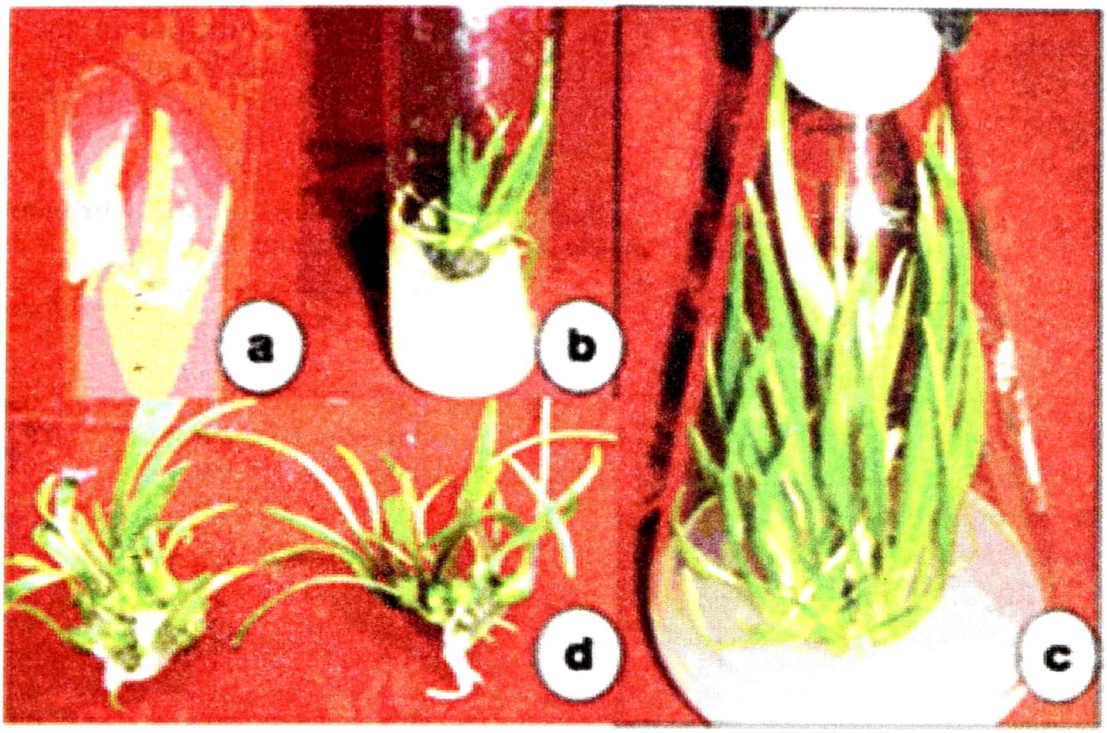

Figure 1. Culture initiation (a), shoot bud induction (b) and (c) and rooting (d) due oryzalin treatments in *Lilium*

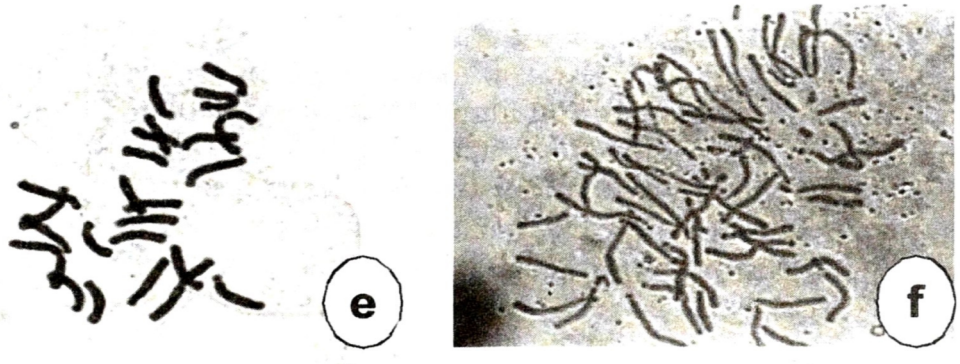

Figure 2. Chromosome counts under microscope (a) Normal diploid (40X) (b) Tetraploid form induced due to oryzalin (40X)

Diploids were found in all the treatments. Diploid shoots resulting from these explants may be the result of regeneration from cells in interior of the explant that was protected from the chemicals. In addition, in the bulb scale segments only a portion of the cells or cell layers may be affected, while the other portion remain diploid (Pryor and Fraiser, 1968). Instead, if single cells are treated, the result is a plant with only one ploidy level.

Table 4. Effect of pre-culture treatment of oryzalin on mitotic chromosomal polyploidization in bulb scales of Asiatic hybrid lily var. Pollyanna.

Concentration (%)	*Treatment duration (hours*	*Number of test plants.*	*Ploidy (%)*			*Efficiency*[a]
			Diploid	*Tetraploid*	*Mixoploids/ Aneupolids*	
0.00	2	11	100.0	0.0	0.0	0.0
	4	11	100.0	0.0	0.0	0.0
	6	11	100.0	0.0	0.0	0.0
0.001	2	33	75.0	25.0	0.0	0.66
	4	40	75.0	25.0	0.0	0.95
	6	30	55.0	45.0	0.0	1.10
0.003	2	26	75.2	24.8	0.0	0.46
	4	16	58.4	31.7	10.0	0.27
	6	15	55.6	33.3	11.1	0.26
0.005	2	16	56.3	18.3	25.4	0.17
	4	10	58.3	20.8	20.8	0.05
	6	3	25.0	0.0	75.0	0.00
0.007	2	18	40.0	11.3	43.6	0.12
	4	8	25.0	25.0	50.0	0.04
	6	8	36.7	10.0	53.3	0.04
0.01	2	6	37.5	0.0	62.5	0.00
	4	3	25.0	0.0	75.0	0.00
	6	2	50.0	0.0	50.0	0.00

[a]Efficiency = Survival rate x No. of shoots per bulbscale x % of tetraploidy/ 1000 (Van Tuyl *et al.*, 1992)

The chromosome doubling effect of oryzalin is anticipated as these chemical binds strongly to plant tubulins and lead to high microtubule depolymerising activity (Anderson *et al.*, 1991). Cell division is arrested during metaphase and the nuclear membrane is formed again without migration of the chromosomes, leading to polyploidy.

The increasing concentration of oryzalin was recorded with increase in number of unequal chromosome numbers than diploids/tetraploids. The production of mixoploids is due to the lower accessibility of certain pre-existing cells to the doubling agent. The production of 'mixoploid' plants in this study clearly supports the hypothesis of a pluri-cellular origin of *in vitro* regenerated organs in *Lilium*. Chemical acts first on individual cells in the tissue and during the regeneration process, these cells together with unaffected

diploid cells, subsequently contribute to the formation new organs (Chauvin *et al.*, 2005).

The highest efficiency of tetraploid induction (1.1) was recorded from the 0.001 % oryzalin for 6 hrs, followed by 4 hour duration (0.95). The effectiveness in producing polyploids is expressed as the proportion of plants reacting to the particular treatment. The low efficiency of tetraploid conversion shows that chemical binding to tubulins is not sufficient at these concentrations and durations used in these experiments. The most effective treatment concentration is comparable to those reported by other workers (Vainola, 2000; Cohen and Yao, 1996).

Confirming tetraploids by chromosome number are found to be efficient and accurate (Cohen and Yao, 1996). However, it is a very laborious process when large numbers of plants need to be examined. Chromosome doubling is accepted as a source of evolution of flowering plants and breeders benefit from it for the domestication of certain genotypes as cultivars and for the restoration of inter-specific or inter-generic hybrids (Van Tuyl *et al.* 1992).

CONCLUSIONS

Cytological study of Asiatic hybrid lily var. Pollyanna root tips indicated diploid chromosome number 2n=2x=24. Increasing the concentration of oryzalin reduces explant survival and delayed bud sprouting but concentration and time duration interaction was not significant. A few shoots per explant with stunted growth in the initial stages and delayed rooting with slow growth was found in regenerated chemical treated plants. Exposure to 0.001 % oryzalin for 6 hrs was found to be the best for *in vitro* induction of tetraploid plants in Pollyanna. This technique can be used in breeding and further varietal improvement programmes of the crop.

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