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Polyploidy induction through colchicine and oryzalin in *Bougainvillea peruviana* cv. Shubhra and assessments of variation through DUS characterization

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Abstract
The objective of this study was to assess the effectiveness of colchicine and oryzalin in inducing polyploidy in *Bougainvillea peruviana* cv. Shubhra. Colchicine was administered at concentrations ranging from 0.1% to 0.5%, and oryzalin was applied at concentrations ranging from 50 to 200 µM, with treatment durations spanning from 48 to 96 hours. To assess the polyploidisation flow cytometry was employed to screen the ploidy levels of the treated plants. Colchicine was more efficient than oryzalin in terms of polyploidy induction, leading to notable variations in the vegetative characteristics of Bougainvillea.

Keywords: Polyploidy, flow cytometer, colchicine, oryzalin, DUS

Introduction
The ever-evolving preferences of consumers in the floriculture trade consistently drive the need for introducing new varieties. This necessitates the induction of genetic alterations, a crucial prerequisite for the development of novel forms that play a pivotal role in the field of breeding. Polyploidy, defined as the heritable condition of having more than two complete sets of chromosomes (Comai 2005) [3], holds significant importance in shaping genetic and phenotypic diversity, influencing both plant evolution and breeding strategies (Xing *et al.* 2011) [16]. Polyploidy breeding is an effective method for creating genetic variation by doubling the chromosome number of any species. These new forms with improved plant architecture provide good material for breeding programme and for further development of cultivars (Mata, 2009) [8]. Bougainvillea is a captivating shrub renowned for its vibrant colours, it graces gardens and homes, adding a spectacular touch to the surroundings. Belonging to the Nyctanginaceae family and native to South America, this plant is not only incomparable in beauty but also remarkably versatile in its applications. It was first noted by the French navigator Louis Antoine de Bougainville in Brazil in 1768. Bougainvillea exhibits unparalleled beauty with its mass of brightly coloured bracts, making it one of the most floriferous shrubs. Its utility extends beyond aesthetics, as it is employed in mass plantings, as shrubs or bushes, and as ground cover on banks. The plant serves various purposes, including providing hedges, barriers, and slope coverings. The popularity of bougainvillea is largely due to its adaptability to different usage besides its ability to change the surroundings dramatically in colourful and purposeful manner (Sindhu *et al.*, 2012) [13]. It is used as an accent plant, a specimen plant, in hanging baskets, in containers, and for bonsai (Kobayashi *et al.*, 2007) [7]. Chemicals like colchicine, oryzalin, trifluralin, amiprophosmethyl (APM), etc., are commonly used in induction of polyploidy. Colchicine is extensively used for induction of polyploidy in plants, oryzalin is also used for polyploidy induction for its less toxic nature. Concentration and duration of treatment of the anti-mitotic agents affects the success percentage in polyploidy induction. The use of colchicine as a means of chromosome doubling has opened a large reservoir of possibilities in plant breeding work. Most conspicuous morphological effects of induced polyploidy includes increase in size and shape of plants; leaves, branches, flower parts, fruits and seeds (Chopra, 2008) [2]. Considering the effectiveness of polyploid breeding as a tool for generating variation in vegetatively propagated crops, and recognizing the limited research on polyploidization in Bougainvillea, the current research study was undertaken.

Materials and Methods
Hardwood cuttings of Bougainvillea cv. Shubhra were obtained from the Research farm of

International Centre of Registration Authority (ICRA) for Bougainvillea, ICAR-IARI, New Delhi, India and were planted in polybags and kept in mist chamber for rooting. Immediately after the emergence of dormant buds in the nodal regions, cotton plugs saturated with different concentrations of colchicine (0.1, 0.2, 0.3, 0.4 and 0.5%) and oryzalin (50 µM, 100 µM, 150 µM and 200 µM) solutions were placed intact on the sprouts for a period of 48, 72 and 96 hours. The cotton plugs were resoaked with the chemicals when required. The treated cuttings were covered using polycaps to avoid contact with water.

DNA ploidy of treated cuttings was determined by using Sysmex CyFlow™ Cube 6 Flow cytometer which is equipped with 2 light sources, using a 488nm blue laser or a 638nm red laser with a maximum acquisition rate at 15,000 particles per second as per the protocol described by Eeckhaut *et al.*, 2005 [5]; Fomicheva and Domblides, (2023) [6] by using flow cytometry (FCM) analysis. The actively growing, around 20 mg youngest leaf tissues were collected for analysis and were chopped by a sharp razor blade in a small petridish in presence of 1ml staining solution (Sysmex Cystain UV Ploidy) and then the nuclear suspensions were filtered through a 30 µm filter (CellTrics) in an eppendorf tube and the clear filtered suspension was obtained. The filtered suspension was then subjected to the FCM for determining the ploidy status. Histograms were analysed using the internal CyFlow software of the FCM, which determines peak position and the relative ploidy index of the samples. The variations in the putative polyploids were analysed as per DUS characterisation given by PPV & FRA. The entire experiment was carried out in factorial completely randomized design (CRD) with two factors making it a combination of thirty treatments, three replications per treatment and five plants per replications.

Results and Discussion

The survivability percent of treated cuttings decreases with high concentration as well as longer duration of colchicine and oryzalin exposure. Notably, colchicine at a concentration of 0.5% when exposed for a period of 72 hours yielded the highest mortality rate, resulting in 0% survivability of the cuttings, whereas when the cuttings were treated with 0.2% colchicine for 72 hours it showed maximum survivability (Table 1). Elevated levels of colchicine have been linked to the demise of plant cells, attributed to its highly toxic impact, impeding spindle fiber development, and altering the differentiation process (Pintos *et al.*, 2007) [11]. The fluctuation in ploidy levels under identical treatment conditions might be attributable to the cellular stage responsible for instigating new shoots (Thao *et al.*, 2003) [14].

Table 1: Effect of anti-mitotic agents on the survivability (%), internodal length and thorn length of *Bougainvillea peruviana* cv Shubhra

Treatment/ Duration	Survival Percent (%)			Internodal Length (cm)			Thorn Length (cm)		
	48 hrs	72 hrs	96 hrs	48 hrs	72 hrs	96 hrs	48 hrs	72 hrs	96 hrs
T_1: Control	50.01^{e}	40.01^{f}	33.33^{g}	1.71^{ab}	1.66^{abcd}	1.83^{a}	0.83^{bcde}	1.14^{ab}	1.18^{a}
T_2: 0.1% colchicine	60.01^{d}	75.01^{b}	60.00^{d}	1.54^{bcdef}	1.58^{bcdef}	1.57^{bcdef}	0.77^{cdef}	0.64^{cdef}	0.57^{cdef}
T_3: 0.2% colchicine	28.57^{h}	90.00^{a}	33.34^{g}	1.52^{cdefg}	1.48^{defg}	1.57^{bcdef}	0.63^{cdef}	0.65^{cdef}	0.88^{abcd}
T_4: 0.3% colchicine	40.00^{f}	50.00^{e}	28.57^{h}	1.42^{fg}	1.47^{efg}	1.52^{cdef}	0.84^{bcde}	0.65^{cdef}	0.83^{bcde}
T_5: 0.4% colchicine	50.01^{e}	33.34^{g}	20.00^{j}	1.57^{bcdef}	1.34^{g}	1.55^{bcdef}	0.47^{f}	0.71^{cdef}	0.55^{ef}
T_6: 0.5% colchicine	16.67^{k}	0.00^{m}	33.34^{g}	1.47^{efg}	0.00	1.65^{abcde}	0.72^{cdef}	0.00	0.56^{def}
T_7: 50 µM oryzalin	40.00^{f}	25.01^{i}	50.00^{e}	1.48^{defg}	1.50^{cdefg}	1.55^{bcdef}	0.89^{abc}	0.76^{cdef}	0.86^{abcde}
T_8: 100 µM oryzalin	20.00^{j}	40.00^{f}	50.00^{e}	1.54^{bcdef}	1.61^{bcdef}	1.68^{abc}	0.64^{cdef}	0.68^{cdef}	0.59^{cdef}
T_9: 150 µM oryzalin	11.11^{l}	66.67^{c}	20.00^{j}	1.57^{bcdef}	1.59^{bcdef}	1.67^{abcd}	0.83^{bcde}	0.76^{cdef}	0.74^{cdef}
T_{10}: 200 µM oryzalin	11.11^{l}	33.34^{g}	16.67^{k}	1.64^{bcde}	1.54^{bcdef}	1.60^{bcdef}	0.65^{cdef}	0.62^{def}	0.62^{cdef}
S.Em± Duration	0.225			0.036			0.036		
Treatment	0.410			0.065			0.066		
D x T	0.710			0.113			0.114		
CD Duration	0.636			0.101			NS		
Treatment	1.160			0.184			0.186		
D x T	2.010			0.318			0.323		

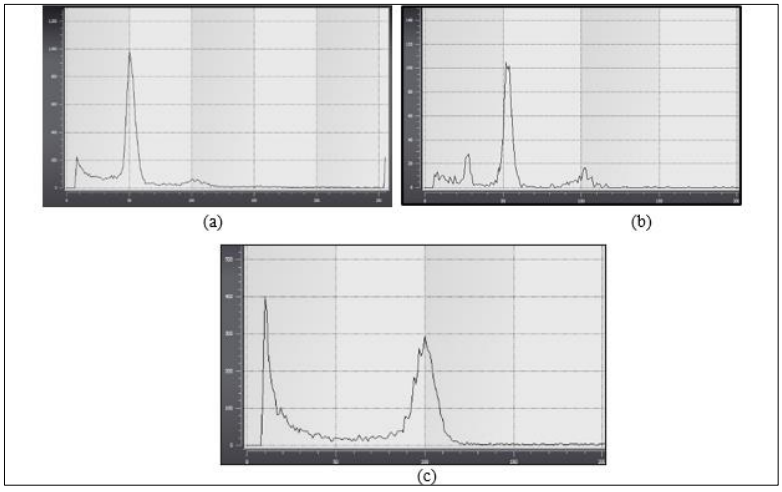

Fig 1: Histogram of relative DNA content of nuclei isolated from diploid (a), tetraploid (b) and mixoploid (c) Bougainvillea cv Shubhra. Diploid (control) nuclei were set to channel 50 with tetraploids resolving at channel 100 and mixoploid resolving both channels.

Based on the peak of the histograms in flow cytometer ploidy level of the treated cuttings were obtained diploid or control nuclei were set to channel 50 with tetraploids resolving at channel 100 and mixoploid resolving at both channels. When the survived plants were subjected to flow cytometer, two plants were found to be putatively polyploids, two mixoploid plants were obtained when they were treated with 0.3% colchicine for 48 and 72 hours and tetraploid plant was obtained when they were treated with 0.4% colchicine for a period of 96 hours. According to Morejohn *et al.,* (1987) [9] elevated concentrations of colchicine required for inducing polyploidy, primarily due to the poor binding of colchicine to plant tubulins. While numerous cells in meristematic tissue may undergo polyploidization, others may remain unaffected, retaining their diploid state. Consequently, regions where both normal and polyploidized cells coexist are termed 'mixoploidy' or colloquially referred to as 'chimera' (Dermen and Henry, 1944; Pryor and Frazier, 1968; Wan *et al.,* 1989) [4, 12, 15]. The prevalence of chimeric plants primarily stems from the type of tissue employed. In multicellular buds or meristems, only specific cells or layers may undergo changes, leaving the remainder diploid (Pryor and Frazier, 1968) [12]. Efforts were made to induce polyploidization in bougainvillea using colchicine and oryzalin; however, the treated plant materials did not show economically significant improvements in performance. The internodal length was found to be moderate, while the thorns exhibited elongated, straight characteristics with a medium density and shorter length when treated with 0.4% colchicine for 48 hours (Table 1 and Figure 2).

Table 2: Effect of anti-mitotic agents on the leaf blade length, width and petiole length of *Bougainvillea peruviana* cv Shubhra

Treatment/ Duration	Leaf Blade Length (cm)			Leaf Blade Width (cm)			Petiole Length (cm)		
	48 hrs	72 hrs	96 hrs	48 hrs	72 hrs	96 hrs	48 hrs	72 hrs	96 hrs
T_1: Control	7.57^{a}	7.03^{abc}	6.67^{abcd}	3.80^{bcdefg}	3.47^{efgh}	4.07^{abcd}	1.50^{abc}	1.17^{cde}	1.16^{cde}
T_2: 0.1% colchicine	7.23^{ab}	6.5^{abcd}	5.67^{cd}	3.27^{gh}	3.97^{abcde}	3.60^{defgh}	1.34^{bcde}	1.10^{de}	1.30^{bcde}
T_3: 0.2% colchicine	6.47^{abcd}	6.23^{abcd}	5.63^{cd}	3.73^{cdefgh}	3.60^{defgh}	3.37^{fgh}	1.10^{de}	1.27^{bcde}	1.24^{cde}
T_4: 0.3% colchicine	5.37^{d}	7.23^{ab}	6.53^{abcd}	4.3^{ab}	4.17^{abc}	4.43^{a}	1.07^{e}	1.17^{cde}	1.10^{de}
T_5: 0.4% colchicine	5.53^{d}	7.27^{ab}	6.77^{abcd}	3.23^{h}	4.13^{abcd}	3.50^{efgh}	1.17^{cde}	1.27^{bcde}	1.44^{abcde}
T_6: 0.5% colchicine	6.5^{abcd}	0.00	6.77^{abcd}	3.23^{h}	0.00	3.37^{fgh}	1.63^{ab}	0.00	1.54^{abc}
T_7: 50 µM oryzalin	6.23^{abcd}	6.47^{abcd}	7.2^{ab}	3.5^{efgh}	3.37^{fgh}	3.60^{defgh}	1.07^{e}	1.24^{cde}	1.17^{cde}
T_8: 100 µM oryzalin	7.3^{ab}	5.87^{bcd}	6.57^{abcd}	3.43^{efgh}	3.4^{fgh}	3.43^{efgh}	1.47^{abcd}	1.47^{abcd}	1.733^{a}
T_9: 150 µM oryzalin	6.63^{abcd}	6.3^{abcd}	7.57^{a}	3.33^{fgh}	3.27^{gh}	3.87^{bcdef}	1.24^{cde}	1.3^{bcde}	1.30^{bcde}
T_{10}: 200 µM oryzalin	7.03^{abc}	6.63^{abcd}	6.23^{abcd}	3.37^{fgh}	3.47^{efgh}	3.43^{efgh}	1.24^{cde}	1.37^{abcde}	1.24^{cde}
S.Em ± Duration	0.163			0.063			0.042		
Treatment	0.298			0.116			0.077		
D x T	0.516			0.200			0.134		
CD Duration	0.462			0.179			0.120		
Treatment	0.843			0.327			0.219		
D x T	1.461			0.567			0.379		

The leaf length and width were smaller when the cuttings were treated with higher concentrations of colchicine and oryzalin (Table 2). Some plants showed variations in their vegetative phase, originally cv. Shubhra have upright type of growth habit but when it was treated with 100 µM oryzalin for 48 hrs it showed spreading type of growth. Similarly, the young leaf colour of the treated cuttings were dark green instead of light green colour in many of the cuttings when they were treated with 0.1%, 0.3% colchicine for 72 hrs and with 0.4% colchicine for 48 hrs (Figure 2).

Table 3: Effect of anti-mitotic agents on the Inflorescence length, flower diameter and peduncle length of *Bougainvillea peruviana* cv Shubhra

Treatment/ Duration	Inflorescence Length (cm)			Flower diameter (cm)			Peduncle length (cm)		
	48 hrs	72 hrs	96 hrs	48 hrs	72 hrs	96 hrs	48 hrs	72 hrs	96 hrs
T_1: Control	27.85^{bcd}	29.77^{ab}	31.82^{a}	0.63^{cdefgh}	0.63^{cdefgh}	0.63^{cdefgh}	6.25^{a}	6.07^{ab}	5.12^{bc}
T_2: 0.1% colchicine	25.75^{cd}	26.43^{cd}	26.40^{cd}	0.65^{abc}	0.63^{bcdefg}	0.64^{abcde}	5.94^{abc}	4.97^{c}	5.10^{bc}
T_3: 0.2% colchicine	27.65^{bcd}	26.02^{cd}	27.07^{bcd}	0.60^{hi}	0.61^{defgh}	0.60^{hi}	5.29^{abc}	5.58^{abc}	5.43^{abc}
T_4: 0.3% colchicine	26.02^{cd}	24.95^{d}	25.51^{cd}	0.61^{defgh}	0.62^{cdefgh}	0.64^{abcd}	5.36^{abc}	5.37^{abc}	5.50^{abc}
T_5: 0.4% colchicine	26.42^{cd}	25.71^{cd}	26.24^{cd}	0.62^{cdefgh}	0.65^{abc}	0.62^{cdefgh}	5.43^{abc}	5.70^{abc}	5.96^{abc}
T_6: 0.5% colchicine	27.93^{bcd}	0.00^{e}	28.14^{bc}	0.64^{abcde}	0.00^{j}	0.67^{a}	5.56^{abc}	0.00^{d}	5.20^{bc}
T_7: 50 µM oryzalin	26.38^{cd}	26.83^{bcd}	25.82^{cd}	0.61^{fghi}	0.61^{fghi}	0.64^{abcdef}	5.68^{abc}	5.49^{abc}	5.41^{abc}
T_8: 100 µM oryzalin	25.94^{cd}	26.07^{cd}	26.11^{cd}	0.63^{bcdefg}	0.66^{ab}	0.61^{efgh}	5.76^{abc}	5.58^{abc}	5.70^{abc}
T_9: 150 µM oryzalin	25.20^{cd}	25.20^{cd}	25.77^{cd}	0.63^{cdefgh}	0.61^{defgh}	0.58^{i}	5.22^{bc}	5.67^{abc}	5.64^{abc}
T_{10}: 200 µM oryzalin	27.17^{bcd}	28.09^{bc}	26.77^{bcd}	0.61^{defgh}	0.61^{fghi}	0.60^{ghi}	5.63^{abc}	5.94^{abc}	5.79^{abc}
S.Em± Duration	0.339			0.004			0.114		
Treatment	0.619			0.007			0.207		
D x T	1.072			0.012			0.359		
CD Duration	0.959			0.011			0.321		
Treatment	1.750			0.019			0.587		
D x T	0.301			0.033			1.016		

Polyploidy normally results in thicker foliage, a richer green hue, a heightened width-to-length ratio of leaves, larger and intricately textured flowers, and a more condensed growth pattern (Amir *et al.,* 2010) [1]. Leaf blade shape and the shape of apex was different when they were treated with 100 µM oryzalin for 48 hours, they exhibited dissected structure

instead of normal acuminate type to structure, these findings were similar to the results of Omidbaigia *et al*., (2010) [10]. Other than the variations depicted in graph (Figure 2) there were no differences in other morphological characters when the cuttings were treated with different concentrations of colchicine and oryzalin for different durations.

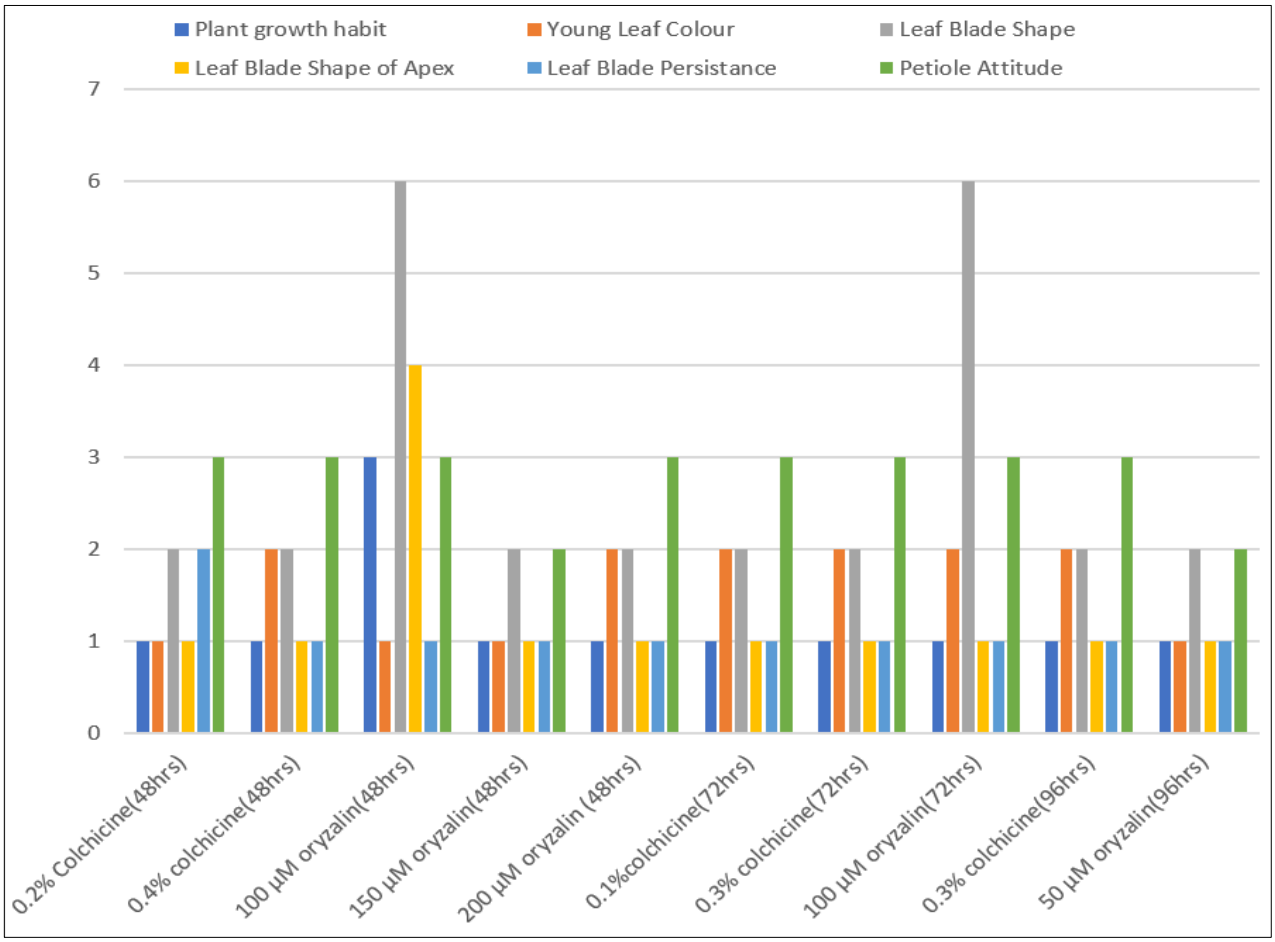

Fig 2: Effect of different concentration of colchicine and oryzalin when treated for different duration on the morphological characterstics of bougainvillea cv Shubhra.

Conclusion

The successful induction of polyploids in bougainvillea validates the efficacy of colchicine and oryzalin as polyploidizing agents. To achieve a higher frequency of chromosomal changes, it is essential to standardize the concentrations, exposure time, and application method of colchicine for each specific plant species. The polyploids acquired will be evaluated further and employed in subsequent breeding endeavors.

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