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Role of polyploidy in orchid improvement

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Abstract
Orchids are the most beautiful flowers and unique group of plants in nature. They are consistently ranked among the best sellers in the global potted plant trade. Today orchids dominate the cut flower and potted plant commerce due to their long-lasting charm, high productivity, seasonal blooming, convenient packing and transportation. Polyploidy can be induced in orchids using antimitotic agents by exploiting the endopolyploid cells or without using it, via *in vivo* and *in vitro* techniques. Identification of polyploids can be performed by examining physiological and morphological traits, chromosome counting and flow cytometry. Polyploidy contributes for improvement of several characters in orchids like flower size and shape, aesthetic value, metabolite content, stress tolerance, better ion utilization capacity and development of new hybrid.

Keywords: Antimitotic agents, endopolyploidy, flow cytometry, *in vitro*, *in vivo*, orchids, polyploidy induction, protocorm like bodies (PLBs)

Introduction
Orchid belongs to Family Orchidaceae, is the largest and highly evolved family, accounting for almost 10% of flowering plants, and comprises about 35,000 species belonging to 850 genera. Orchids have high economic value in commercial horticulture, as potted plants or cut flowers, and in traditional Chinese medicine (Grosso *et al*., 2017) [11].
Orchids are the most beautiful flowers and unique group of plants in nature. They are consistently ranked among the best sellers in the global potted plant trade (Hinsley *et al*., 2018) [13]. Orchid cultivation is now an international business covering around 10% of the world floriculture trade (Sarmah *et al*., 2017) [28]. Orchids dominate the cut flower and potted plant commerce due to its long-lasting charm, high productivity, right seasonal blooming, convenient packing, and transportation (De *et al*., 2014) [5].
There are several methods for improving a crop that includes conventional, non-Conventional and advanced breeding techniques and polyploidy breeding is one among them.

Polyploidy
Polyploids are organisms which are having one or multiple sets of chromosomes in addition to their normal diploid number (Ramsey and Schemske 1998) [25]. It is common in nature, which can be considered as a mechanism for speciation and adaptation. It has been reported that 50-70% of angiosperms have undergone polyploidy during their evolutionary process. (Chen *et al*., 2007) [2]. The mechanisms behind the polyploidy includes; somatic doubling during mitosis, non-reduction during meiosis which leads to the production of unreduced gametes, polyspermy (fertilization of the egg by two male nuclei) *etc.* (Grant, 1981) [9].

Techniques of inducing polyploidy
There are several methods for inducing polyploidy *viz*., decapitation, graft combination, radiation, hybridization, temperature treatment, sexual polyploidization and by using antimitotic agents or chemicals. Among this, chemically induced polyploidy is found to be more effective. Ranney (2006) [26], suggested that for the doubling of the chromosome, seedlings or apical meristems can be made use of by either soaking or submerging in different
Concentrations of doubling agent for a particular duration. Colchicine and oryzalin are the among the most widely used antimitotic agents for the induction of polyploidy in plants (Sattler *et al*., 2016) [29]. Apart from colchicine and oryzalin, other chemicals include, colchamine, colcemid, trifluralin, amiprophosmethyl etc. The effectiveness of colchicine application and polyploidy induction depends on several factors like, the type of explant, time of exposure of these explants to the colchicine and also the concentration of colchicine. (Zakizadeh *et al*., 2020) [41].

Colchicine is an alkaloid which is extracted from the meadow saffron (*Colchicum autumnale* L.) and used as an antimitotic agent for polyploidy induction. The mechanism by which colchicine performs the antimitotic activity involves its binding to α- and β-tubulin dimers resulting in inhibition of the microtubule polymerization during the cell cycle event and thus preventing the chromosome/chromatid from migrating towards the poles during the anaphase cell stage. As a result, cytokinesis gets compromised, leading to the formation of cells with doubled chromosome number (Planchais *et al*. 2000) [24]. The consequences of polyploidy include genome buffering, gigas effect, heterosis, heterozygosity, genetic and epigenetic changes, fertility restoration, low fertility and seedless fruits (Sattler *et al*., 2016) [29]. *al*. 2007) [7]. The peak position of the flow cytometry histogram of unknown sample and the control sample can be compared, in order to confirm the ploidy manipulation in the treated samples.

Polyploidy in orchids

Polyploid orchids has been established to be generally superior to their diploid counterparts, and thus the artificial induction of polyploidy assumes particular significance as, superior horticultural varieties might be obtained immediately, if somatic doubling can be induced using colchicine. Such tetraploids can be utilized in breeding of further polyploids (Nakasone, 1960) [21].

Polyploid orchids, like many other polyploid plants, produce larger flowers than their diploid relatives. In cutflower trade, large size and heavy substance are considered as highly favourable characteristics along with the longer shelf life (Chaicharoen and Saejew, 1981) [1].

Identification of polyploids

The methods for identification of polyploid individuals include direct and indirect methods. Indirect methods involve the examination of physiological traits especially those related with stomata and/or morphological traits i.e., based on the morphological changes exhibited by a polyploid plant and its diploid counterpart. On comparing the diploid relatives with polyploidy plants, the polyploids usually display large sized stomata but with a lower density, and the number of chloroplasts per guard cell shows a higher value. Such stomata features have been efficiently used to distinguish the polyploidy regenerants of several plant species in orchids (Silva *et al*. 2000) [31]. Though the indirect procedures for polyploidy detection are usually rapid and simple, they are often inaccurate and thus, the confirmation through direct methods, such as chromosome counting using the standardized staining techniques and nuclear genome size measurement by flow cytometry, is usually necessary. In the flow cytometry analysis, the analysis is performed with an assumption that an increment in the DNA content corresponds to an increment in chromosome number. The DNA content of an exemplar with a known ploidy level can be used as a reference standard to determinate the DNA ploidy level of an unknown sample (Dolezel *et* Breeding program in orchids encountered relatively high degree of sterility among intergeneric hybrids and to a lesser degree among interspecific hybrids. Sterility among diploids result from incompatibility of genes, male sterile genes, or genes that cause irregularities in meiosis such as supernumerary cell divisions, asynapsis and stickiness. Translocations, inversions, and deletions of chromosomes may also result in sterility. The cytological basis which accounts for such sterility in orchids is mainly due to nonhomology of the chromosomes. It has been well demonstrated in plants that this type of sterility barrier could be removed by the use of colchicine and thus, doubling the chromosome numbers of the sterile types. Restoration of fertility among the sterile hybrids was found desirable in order to advance the breeding program (Kamemoto 1950; Storey 1952) [14, 32].

Orchid culture has been benefited with the fertility restoration of hybrids with problems of chromosome pairing and assortment during meiosis. It contributed for increase in the genetic variability. The induced polyploids resulted in flowers which are usually larger in size, with rounder conformation, and greater substance than the diploids (Wimber and Wimber 1967) [39].

Breeding behaviour of polyploid and aneuploid orchids

Triploids are generally of low fertility and often represented a dead-end in breeding programme. The reason behind the poor fertility is the high irregularity in reduction division. Because there are three sets of chromosomes, distribution of chromosomes to the poles are unequal, resulting in pollen and eggs with varying chromosome numbers, and many of which are non-functional. Occasionally, restitution of nuclei will lead to functional unreduced eggs and pollen. Neverthless, some triploids also produce offspring. The chances of success of triploids are improved if they are being used as the seed-bearing parent instead of the pollen parent, and use of tetraploids as the pollen parent instead of diploids. Eg. The cross between triploid *Dendrobium* Lady Constance X Diploid *D. phalaenopsis* has resulted in production of seedlings with chromosome numbers 38, 42, 46, 51, 52, 57, and 75. This type of cross has the chance of producing pentaploid, tetraploid, triploid and diploid offspring in addition to aneuploids. (Kamemeto *et al*., 1961) The best orchid plants are tetraploids because they are fertile and also produce offspring that are relatively uniform. Variations in degree of fertility among tetraploids depends on the constitution of the chromosome complements. If all four sets are found to be uniform, such as in the case of Vanda, tetraploid strap-leaved (autotetraploid, SSSS), fertility may be reduced due to the irregularity in meiosis which may be due to the formation of univalents, bivalents, trivalents, and tetravalents. On the other hand, Vanda, tetraploid semiterete (allotetraploid, SSTT) exhibits less irregularity during meiosis because normal chromosome pairing can happen within similar sets of chromosomes of terete and strap Vanda. To produce further tetraploids, tetraploids can be selfed or crossed with other tetraploids. When it is crossed with diploids, it will result in triploids; while crossing with triploids, variable offspring might be obtained. (Kamemeto *et al*., 1961) [15]. Pentaploids are generally found to be more fertile than triploids. The two sets of chromosomes reach either pole or the chromosomes of the extra set assort at random was observed in the meiosis of pentaploid strap-terete Vanda. (Kamemeto *et al*., 1961) [15]. Aneuploid Cattleyas having 61 or 62 chromosomes would show poor fertility similar to the triploids having 60 chromosomes, while the aneuploids having 81 or 82 chromosomes can be expected to show fertility similar to tetraploids having 80 chromosomes. While those aneuploids with chromosome numbers more or less intermediate between the tetraploid and triploid levels can be expected to show low fertility because of chromosome imbalance (Kamemeto *et al*., 1961) [15].

Tracing the history of polyploid orchids

The first report of induced polyploidy in orchids was done by Macleod in 1947. He reported delayed flowering and two sizes of embryos in selfed seeds of treated flowers. In *Laelia anceps* he reported that the treated flowers were twice the normal size and had intensified color.

Moore (1947) [20] while working on *Cattleya trianaei* var. *alba*. reported that leaves of some colchicine treated plants were rough and wrinkled and the plant seemed to have increased its blooming capacity.

Hagerup (1947) [12] observed polyspermy as infrequent phenomenon in orchids. Based on cytological examination of ovules after pollination, he was able to show a single incidence of *in vivo* gamete attachment and/or plasmogamy involving two sperm cells and an egg cell. It remains unclear, however, whether these sperm nuclei also undergo karyogamy with the egg nucleus and what is the fate of this atypical fertilization event.

Chaicharoen and Saejew (1981) [1] reported that polyploidy can be expected to arise spontaneously in nature and during tissue culturing, but treatment with colchicine increases the number of polyploid plants considerably. In their experiment they examined thirty treated seedlings regenerated from the diploid stock, out of which 15 were tetraploids or near tetraploid, 1 aneuploid, 2 octoploids and 12 diploids in *Dendrobium* sp. The plants produced under colchicine treatment had considerable changed characteristics. The growth of tetraploid plants was slower and had greener leaves than their diploid counterparts. Tetraploid plants had thicker leaves. Similar results were found when the guard cell pairs of these leaves were compared.

Polyploidy plays a major role in the development of *Phalaenopsis* varieties. Among the many popular commercial hybrids, most of them are tetraploids. However, on analysis of the pedigree of these hybrids that were developed, it indicated a narrow genetic base. It has been observed that, it is difficult in transferring favorable genes of the diploid wild species to the commercial hybrids that are developed because of their difference in the ploidy levels. The size of the chromosome of Phalaenopsis species varied tremendously which also contributed for their difficulty in breeding programme. Hybrids that were developed from the crosses between these species were usually found to be sterile because of the problem of genome homology. Therefore, inorder to overcome all these barriers and for restoration of fertility in these hybrids, chromosome doubling is one of the methods that can be adopted (Chen *et al.*, 2010) [4].

The series of "Big White Flower" hybrids of *Phalaenopsis*, which are popular in the market, are tetraploids. Development of these hybrids was related to the polyploidization of one of the ancestors, *P.* Doris, which is the most important parent in this group of hybrids. (Tang and Chen, 2007) [34].

Induction of polyploidy in orchids

Polyploidy can be induced in orchids without using antimitotic agents by exploiting the endopolyploid cells. It can also be induced using antimitotic agents via *in vivo* and *in vitro* technique. *In vitro* colchicine induction can be done in cultures from stem nodal, seeds, axillary buds, pseudobulb, transverse thin cell layer from protocorms, leaves explants. *In vivo* technique can be carried out by using small plantlets of orchids, plant cuttings etc.

It has been reported that protocorms of *Cymbidium* (Wimber and Van cott, 1966) [38], *Dendrobium* (Sanguthai *et al.*, 1973) [27],

Phalaenopsis (Griesbach, 1981) [10] and *Paphiopedilum* (Watrous & Wimber, 1988) [37] can be induced to double their chromosome number when treated with colchicine containing liquid culture media and thus tetraploid plants can be regenerated. The concentration of the antimitotic agent as well as the duration of treatment are important factors that should be determined for each type of material for induction of polyploidy (Derman, 1940) [6]. Higher concentrations of drug or prolonged treatments may be lethal to sensitive plant tissue. Chen *et al.* (2011) [3] developed an effective and simple protocol for the production of polyploids by sectioning of the protocorms or protocorm-like bodies (PLBs) without using anti-microtubule agents in eight *Phalaenopsis* species including *P. aphrodite* and *P. bellina.* For that a database for endopolyploidy in different tissues of *Phalaenopsis* species was thoroughly created and studied with the help of flow cytometry analysis. It was found that different patterns of endopolyploidy occurred in different tissues of *Phalaenopsis* species at various stages of development. A large number of stable polyploids were produced through repeated sectioning and regeneration of PLBs from the advanced cultures. When the morphology was studied there is no significant difference in the width of the leaves of diploid and tetraploid plants. However, the length and the leaf index in the tetraploid *P. hieroglyphica* was significantly shorter and lower than that of the diploid, indicating that differences in leaf size between diploid and tetraploid *Phalaenopsis* is genotype specific. Stomata density was found significantly different and thus could be used as a parameter for distinguishing diploid and tetraploid plants in *Phalaenopsis* species. Though there were improvement in stomatal size, stomatal density of tetraploid was lesser than diploids. This investigation revealed that some flower characteristics were different between diploid and tetraploid plants. The flower stalk length in the tetraploid plant was shorter than that of the diploid plant. Diameter of flower stalk and flower size were larger in the tetraploid plants than in the diploid plants. It was also observed that the petals of the tetraploid flowers were heavier and thus had better texture compared to those of the diploid plants.

In vivo polyploidy induction of *Phalaenopsis amabilis* using colchicine was carried out using fully developed plantlets from *in vitro* grown PLBs was reported by Mohammadi *et al*. (2021) [19]. It was concluded from the study that, in order to confirm the data obtained by FCM (flow cytometry), it is better to apply some morphological, anatomical and cytological parameters particularly stomata characteristics. Polyploidy levels can be precisely identified using a combination of these various methods (Fig. 1)

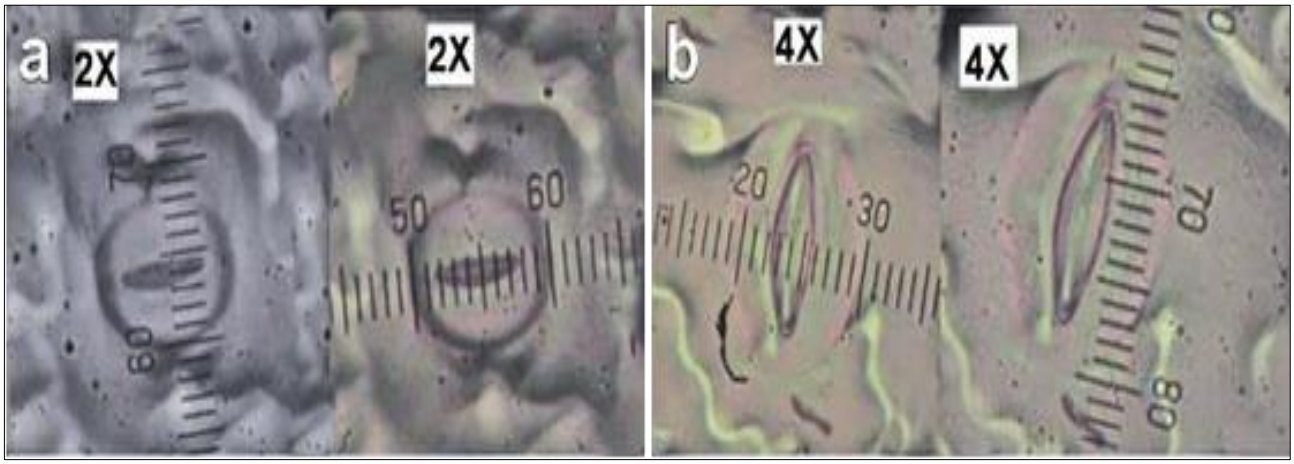

Fig 1: Stomata size (length and width) in diploid (a), and tetraploid (b) of *Phalaenopsis amabilis* var. grandiflora

Vichiato *et al.* (2014) [35] was successful in developing polyploidy in *Dendrobium nobile* Lindl. (Orchidaceae) via *in vivo* method. *D. nobile* diploid (2n = 38 chromosomes) plants with an average height of 5.0 cm were subjected to the treatment. The treatments were based on colchicine concentration (0.5% and 0.1%) and also its duration (24hrs, 48 hrs, 72 hrs, 96 hrs). The first flowering of diploid *D. nobile plants* with normal flowers occurred two years after the conclusion of the polyploidization experiment. It was reported earlier that this species produces flowers in the second year of growth (Neptune, 1984) [22]. *D. nobile* tetraploid plants showed the first flowering with few flowers that had malformed petals, sepals and/ or lips. Seven years after the conclusion of the polyploidy induction experiment, the induced tetraploids of *D. nobile* plants visually had normal flowering. The delay in normal flowering of induced tetraploid *D. nobile* plants might have been resulted from a gene dosage doubling. The immediate effect of polyploidy was morphophysiological i.e., the increased size of the cells due to an increase in nuclear volume. As a result, these cells consume more energy and require more time for the duplication of DNA, which contributes to a reduction of cell division during development, causing a delay in the mitotic cycle and life cycles as expressed in low biomass production per unit time (Takamura and Miyajima 1996, Vichiato *et al*., 2007) [33,36]. The induced polyploidization resulted in decrease in the number of flowers per pseudobulb (40.8%) and also diameter of the pseudobulb (64.9%). It resulted in delayed flowering and showed an increase the number of floral pieces with a greater height of the flower (4.5%), width of the lip (18.5%) and internodes (19.9%). (Vichiato *et al.*, 2014) [35].

Xu *et al.* (2016) [40] developed novel tetraploid *Dendrobium nobile* that tolerates salt stress condition and has greater efficiency in ion utilization by exploiting the polyploidization. Green growing protocorms with similar height obtained from *in vitro* cultures of *Dendrobium nobile* diploid seeds which was cultured on MS media were soaked in medium containing different concentrations of colchicine *viz.* 0.3%, 0.2%, 0.1%. Different concentrations of colchicine were applied to optimize the most effective way to induce the diploid protocorm to the tetraploid lines. Then the tetraploid seedlings were treated by NaCl and KCl for different concentrations for the salt stress test. The ion utilization was measured according to the inductively coupled plasma atomic emission spectrometry (ICP AES). The obtained results demonstrated that after soaking in the solution of 0.2% colchicine, the protocorm got the highest tetraploid induction rate of 33.96%. With the rise in salt concentration, the proliferation index of plants enhanced at first. When the KCl concentration reached 3.70 g/L, the index got to the highest value of 10.97, and then began to decrease. The plants had significant greater tolerability in KCl solution than in NaCl. Compared with the diploid plant, the tetraploid got significantly higher ion utilization rate. In conclusion, this study expanded the germplasm resources of *D. nobile*, which provided a material foundation for improving breeding works of tetraploid and salt tolerant variety with high ion utilization rate.

Grosso *et al.* (2017) [11] manipulated the ploidy level of *Dendrobium* species and developed new varieties having increased ornamental value and a higher secondary metabolite production and confirmed the ploidy through flow cytometry technique. PLBs of 3–5 mm in size were used as explants for polyploidy induction. Stomatal measurements were taken and were compared with diploid hybrid with polyploids. And the result was, the stomatal density of diploid hybrid was higher than the polyploids but the stomatal length is decreased.

Alongwith this, partial duplicated polyploidy was also obtained which had relatively similar stomatal status as tetraploids. The maximum recovery of polyploid platelets was obtained from the concentration of .075% and .025% with 80% and 60% respectively. A large set of negative and positive variations were found in polypoid and initial plantlets regarding metabolite production. But several polyploid genotypes of interest, accumulated higher amount of valuable compounds such as moscatin diacetate and moscatin. There has been increased production of alkaloid like shihuidine (there has been reports indicating its higher Na+/K+ATPase inhibitor activity (Li *et al*. 1991) [16])

In a study conducted by Pham *et al.* (2019) [23] *Dendrobium officinale*, he observed that tetraploid plantlets reported significantly higher polysaccharide contents in leaves, stems, and roots than diploid plantlets. The investigation was carried out by generating a series of polyploid cultivars through colchicine treatment of protocorm-like bodies (PLBs). Tetraploid had shorter leaves, shorter and thicker stems and roots, and they produced higher biomass when compared with the diploid cultivar (Fig 2 and 3). The length and width of stomata increased significantly, but stomatal density decreased in tetraploid cultivars. Polysaccharide contents in stems, leaves, and roots of 6-month-old tetraploid plantlets were significantly higher than those of diploids. The polysaccharide content in the stem of tetraploid was 12.70%, which was a 2-fold increase compared with the diploid cultivar.

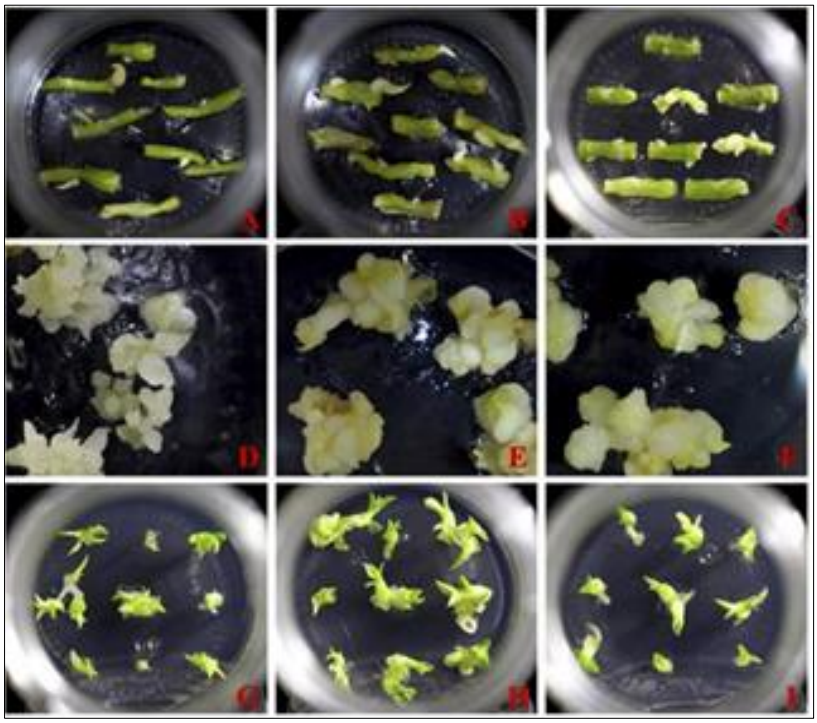

Fig 2: The induction (A–C) and proliferation (D–F) of protocorm-like bodies (PLBs) and plant regeneration (G–I from PLBs of the diploid cultivar (A, D, G) and two tetraploid cultivars (B, E, H) and (C, F, I) of *D. officinale*

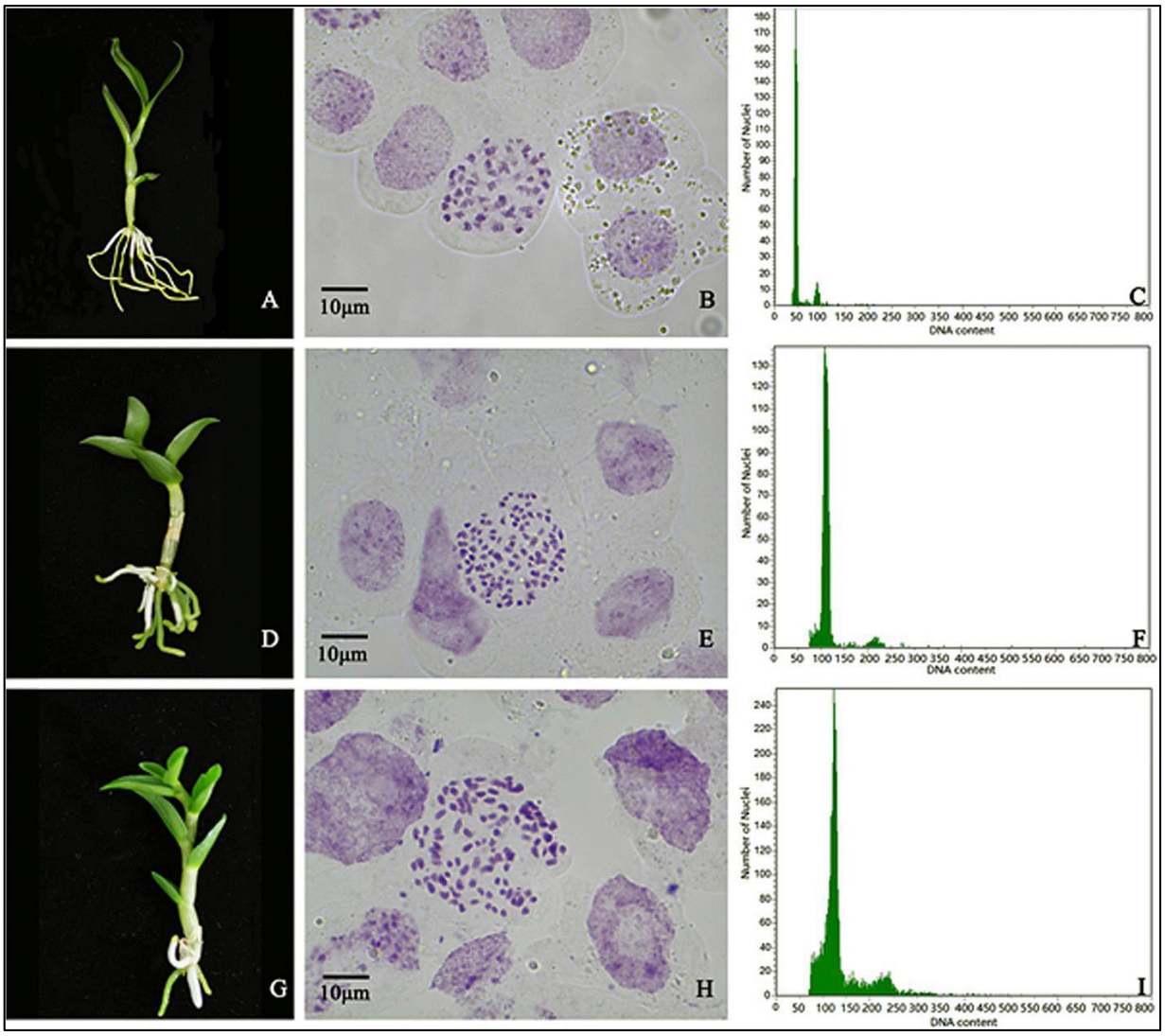

Fig 3: The plantlet appearance, chromosome number in root tip cell, and relative DNA content in leaves of the diploid (2n = 38) parental cultivar (A–C) and two tetraploid (2n = 76) cultivars (D–F) and (G–I) of D. officinale.

Zakizadeh *et al*. (2020) [41], reported a successful method for induction of polyploidy *in vivo* in *Dendrobium* 'Sonia' where the antimitotic agents, colchicine and oryzalin were treated on five-month-old plantlets derived from protocorm like bodies produced *in vitro*.

Disadvantages of polyploidy

On doubling of genome of cell, it is expected to double the volume of space occupied by the chromosomes located within the nucleus, but it resulted in increase in the surface area of the nuclear envelope by 1.6-fold only (Melaragno *et al*., 1993) [18] which affects the normal assumption of the relative changes between the size of the genome and the volume of the cell. This can contribute for the disruption in the balance of factors that are normally involved in mediating the interactions between the nuclear components and chromosomes including the envelope-boundproteins. Sometimes the peripheral positioning of centromeric and telomeric heterochromatin may also be disturbed, as the relative surface space is less on the nuclear envelope in order to accommodate this positioning (Fransz *et al*., 2002) [8].

Polyploidy can also affect the normal completion of meiosis and mitosis, which can create chaotic segregation of chromatids and thus result in the production of aneuploid cells which can lead to abnormal segregation patterns, like 3:1 or 2:1 plus one laggard. It can contribute for the occurrence of un-favorable chimeras. Genetic changes and epigenetic instability can pose yet another challenge for polyploids. Epigenetics are the changes in phenotype and gene expression that are not caused by changes in DNA sequence. According to the genomic shock hypothesis, disturbances in the genome, such as polyploidization, may lead to widespread changes in epigenetic regulation also (Scheild *et al.*, 1996) [30].

Thus, they may explain why induced polyploids do not always reach the initial expectations and overcome their diploid progenitors, or even why tetraploids, for instance, are not twice as productive, vigorous or resistant than their diploid progenitors. Nonetheless, such disturbances have the Potential to produce novel genotypic and phenotypic variations, which may be useful for artificial selection in plant breeding programs**.**

Conclusion

Polyploidy has been extensively studied in the last century and is arguably one of the most important mechanisms of speciation and adaptation in plants. It contributed for improvement of several characters in orchids. Due to betterment in orchid flower quality not only in the size, shape and aesthetic sense but also in its metabolite content, stress tolerance, better ion utilization and also its application in new hybrid development, induction of polyploidy in orchids can be considered as an advantageous technique. It removed sterility barriers and helped in creation of new novel hybrids. Therefore, the application of polyploidy as a tool by plant breeders has allowed the development of increasingly productive and adapted cultivars with good commercial value.

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