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Study on the effect of colchicine on *in-vitro* cultures of turmeric (*Curcuma longa* L.) approach to polyploid development

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Abstract

Crop improvement possibilities in turmeric *Curcuma longa* (L.) are limited due to its triploid nature except for polyploidisation. Colchicine is a chemical that is used frequently to make plants polyploidy. Hence, this research aimed to study the effect of colchicine on the ability to induce polyploidisation of *in-vitro* turmeric cultures. The study consisted of two experiments: (1) assay on *in-vitro* culturing for callus induction and (2) assay on colchicine treatments and polyploidy screening. In experiment 1, turmeric rhizome buds were cultured on MS-solidified medium for callus induction with 100 mL coconut water, 2.5 and 4.5 mg L^{-1} 2,4-D, 0.93 mg L^{-1} KIN, and without growth regulators. In addition, cell suspension culture was tested for callus induction with 4.5 mg L^{-1} 2,4-D and 0.93 mg L^{-1} KIN. For polyploidy induction in experiment 2, *in-vitro* developed callus tissues were transferred to liquid MS medium supplemented with various concentrations of colchicine (0, 0.05, 0.10, 0.15, and 0.20%) for 2 days. Then acetocarmine staining method and microscopic observation were attempted to count the chromosome number. Nucleus size; nucleus area (µmSq) and perimeter (µm) were referred using microscopic observation under 1000× magnification and BEL capture software. The results revealed that MS-solidified medium supplemented with coconut water was most effective in inducing callus. The nucleus area (371.225 µmSq) and perimeter (65.725 µm) of the cells in 0.05% colchicine for 2 days showed the highest results. It can be concluded that 0.05% colchicine concentration has an effect on nuclear size increment thereby possibly inducing polyploidization of turmeric.

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Introduction

Turmeric is regarded as the golden spice with innumerable health benefits. Turmeric, scientifically known as *Curcuma longa* L. belongs to the Zingiberaceae family and genus Curcuma. Turmeric is cultivated most extensively in India, followed by Bangladesh, China, Thailand, Cambodia, Malaysia, Indonesia, and the Philippines. In most tropical regions of Africa, America, and the Pacific Ocean Island, it is also grown on a modest basis. The world's biggest producer, importer, and user of turmeric is India (Shrishail *et al.*, 2013).

It has many cultivars due to its highly variable morphology and the wide range of chromosome numbers in the genus, with diploid, triploid, and tetraploid plants. Curcuma longa belongs to the triploid species (2n=3x=63), a rhizomatous perennial herb whose rhizome is used as one of the most common sources of spices in the world. Turmeric's unique flavour has made it popular for usage as a flavoring ingredient, cosmetic, textile dye, and other applications. Major active ingredients of turmeric include three curcuminoids; curcumin, demethoxycurcumin, and bisdemethoxycurcumin , among curcumin is the main chemical component of turmeric with 0.3-8.6% (Phukan *et al.*, 2022).

Turmeric is a highly valuable plant in the world. Therefore, crop improvement is timely and important for turmeric, targeting high-yielding varieties and enhancing the quality and quantity of curcumin, with high oleoresin and essential oil content, to overcome the hybridisation barrier and enhance the fertility of the plant, pest resistance, environmental adaptability, and stress tolerance for biotic and abiotic stress (Forrester *et al.*, 2020).

Turmeric is propagated by vegetative propagules that sustain the genetic makeup of the crop throughout the generations therefore; the genetic diversity is very low in turmeric. A spontaneous mutation is one way of generating genetic diversity (Ulukapi and Nasircilar, 2018). However, it is a very rare chance to happen (Oladosu *et al.*, 2016). Also, there are many problems when considering conventional breeding of turmeric (*Curcuma longa* L.) and crop improvement (Dudekula *et al.*, 2022). This is a monocotyledonous species, rarely flowering. It is classified as a sterile triploid plant (2n = 3x = 63) and cannot be used as parents for further breeding and to produce sterile flowers with no gametes. Therefore, having drawback of making inter-specific crosses (Ketmaro *et al.*, 2012). Also, during the growing season (8-10 months) each rhizome can produce 10-25 lateral buds, but only 4-6 of them actively develop plantlets. Due to its nature, it has a limited genetic diversity and therefore, crop improvement and conventional breeding is difficult (Upendri and Seran, 2021). Thus a research was aimed to study the effect of colchicine on the ability to induce polyploidisation of in-vitro turmeric cultures.

Materials and methods

The experiment was conducted as a controlled experiment. The experiment design was Complete Randomized Design (CRD) with 4 replicates. The study consisted with two experiments.

Explant preparation and sterilization

In experiment one for assaying in vitro culturing for callus induction, sprouted rhizomes of turmeric (Fig. 1), were obtained from the Export Agriculture Research Center, Matale, Sri Lanka. As Shown in Fig. 1, the rhizomes were washed in running tap water and brushed to remove soil particles and unwanted tissue parts.

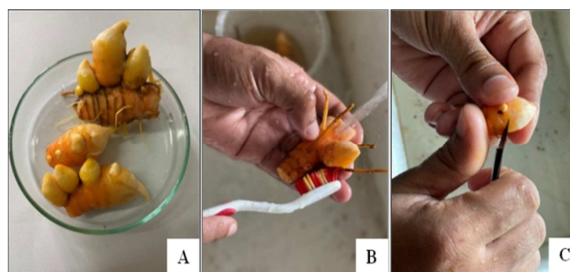

Fig. 1. Sprouted rhizomes of turmeric (A), washing with running tap water to remove the adhering soil particles using a brush (B), and the peeling of rhizome (C)

Peeled sprouted rhizome pieces were washed with Teepol (a detergent) solution and in running tap

water for about 20 min. Then one percent Captan (a fungicide) solution was applied with 2-3 drops of Teepol for 20 min in the orbital shaker and washed 2-3 times with distilled water.

Then sprouted rhizomes were disinfected with 30% surface disinfectant (Clorox) solution with 2-3 drops of surfactant (Tween 20) for 20 min in the shaker and then washed with distilled water for few times to remove the soapiness. Subsequently, the sprouted rhizomes were kept in 50% ethanol for 5-7 min in the shaker washed with distilled water.

Explants transfer to culture media

The 2-3 cm length of shoot buds derived from rhizome were excised using a sterilised scalpel and used as explants.

The explants were cultured on four types of Murashige and Skoog (MS) solid culture media. The media (M1) was without growth regulators, M2 with 2.5 mg L-1 2,4-Dichloropenoxyacetic acid (2,4-D), M3 with 4.5 mg L-1 2,4- D and 0.93 mg L-1 Kinetin (KIN), and M4 with 100 mL coconut water. Separately, callus derived from turmeric shoot buds were cultured in liquid MS medium (M5) supplemented with 4.5 mg L-1 2,4- D and 0.93 mg L-1 KIN and kept on the shaker.

The 2,4- D treated cultures were kept in the dark condition and the rest of the cultures were kept under a photoperiod of 16 hrs. light (2000-3000 lux) and 8 hrs. dark for 3-4 weeks. All cultures were subjected to 20-25°C of temperature and 60%-80% of relative humidity.

In the second experiment, assay on colchicine treatments and polyploidy screening, the callus derived from rhizome buds were cut into small parts and soaked in autoclaved MS liquid medium with different colchicine concentrations (0, 0.05, 0.10, 0.15, and 0.20%) for 2 days under 20-25°C of temperature and with 60%-80% of relative humidity in culture room on the orbit shaker.

Counting of chromosome number in cells

Colchicine-treated calli were washed with sterile distilled water 2-3 times. Then the callus parts were placed on a watch glass and added two drops of 1N HCl for 5-10 minutes. Subsequently, a part of the calli was transferred into to microscopic slide added a drop of 1% acetocarmine. Then the microscopic slide was gently warmed to remove any air bubbles and left for 2-3 minutes. After covering the specimen with a cover slip a folded blotting paper was placed over the coverslip on the slide and pressed firmly on the coverslip with the thumb to prepare a cell layer. Then the chromosomes were counted using a biological light microscope (model: Solaris-T-LED, Bel Engineering) at 1000 × magnification and by using BEL capture software, version 3.2.

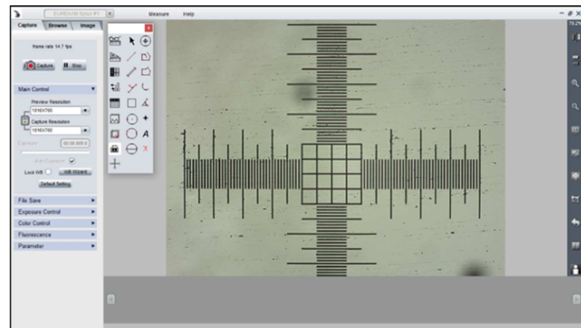

Fig. 2. The main interface of calibration in BEL capture software

Cell Length Width Ratio (LWR) and nucleus size (nucleus area and perimeter) were observed by using a biological light microscope under 1000 × magnification and cell LWR and nucleus size were measured by using BEL capture software (Fig. 2).

Results

In the experiment one, callus development only occurred in M1, M3, and M4. Among them as in Fig. 3, M4 showed the highest callus development percentage (16%) after 6 weeks of culture and M1 showed the lowest callus development percentage (0.769%), after the end of the 6th week (Table 1).

Table 1. Total number of callus development percentage (%) in various MS culture media

Types of MS media	Number of cultures	Total number of cultures (after 8 weeks)			Total number of callus development percentage (%) cultures after	
		Removed	Survived	Dormant	4 weeks	6 weeks
M 1	130	76	54	52	0.769	0.769
M 2	80	64	16	14	0	0
M 3	80	52	28	24	1.25	3.75
M 4	50	17	33	21	4	16
M 5	15	15	0	0	0	0

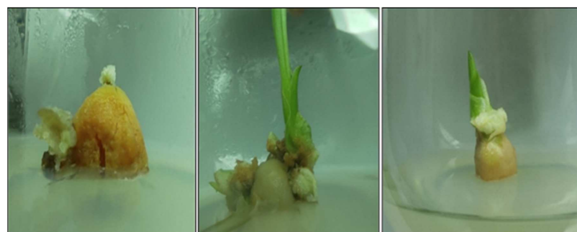

Fig. 3. Turmeric callus in the MS solid culture medium with 100 mL coconut water (M4)

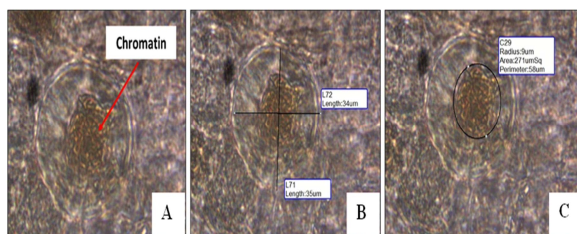

Fig. 4. Microscopically analysed colchicine-treated root tip (A), Method of calculating the cell size (B), and nuclear size (nuclear area and perimeter) (C) by using a biological light microscope under 1000 × magnification and BEL capture software

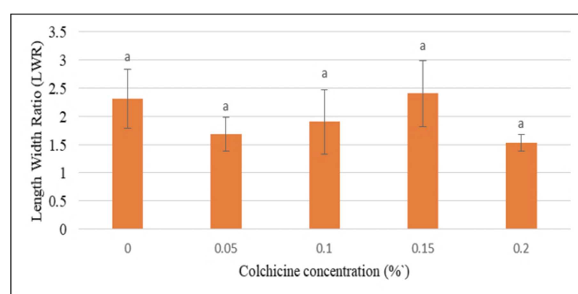

Fig. 5. Relationship between the length-width ratio (LWR) of turmeric cells and different concentrations of colchicine after 2 days

The cell size (length-width ratio), and nucleus size (nucleus area and perimeter) of 10 randomly selected cells were calculated along with the different concentrations of colchicine, and their average values were calculated (Fig. 4) in the experiment two.

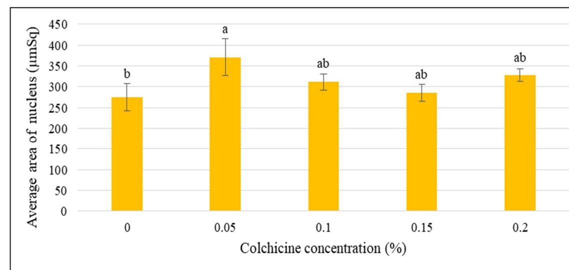

Fig. 6. Relationship between the cell nucleus area of turmeric cells and different concentrations of colchicine after 2 day

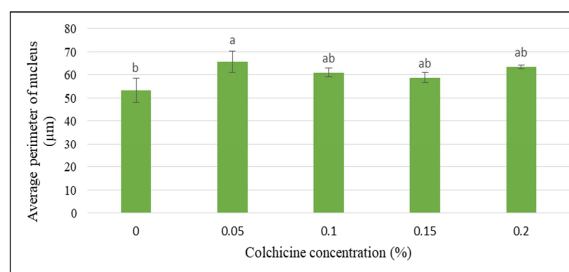

Fig. 7. Relationship between the nucleus perimeter of turmeric cells and different colchicine concentrations after 2 days

According to the result, there is no significant difference among the treatments when considering the length-width ratio of the cell (Fig. 5). The average area and perimeter of the nucleus of colchicine concentration 0 and 0.05% were significantly different from each other and other treatments. The other three treatments did not show significant differences amoung those treatments. The highest average nucleus area (371.225 μmSq) of the nucleus was at 0.05% colchicine concentration (Fig. 6). The highest average perimeter (65.725 μm) of the nucleus was at 0.05% colchicine concentration (Fig. 7).

Discussion

According to the results of the experiment 1, the highest callus growth was observed in media 4 only

which naturally contained concentration of cytokinin, but according to Srivastava *et al.* (2020), Gourguillon *et al.* (2018), Kou *et al.* (2013), it is essential to have high concentrations of auxin or a mixture of auxin and cytokinin. Also, according to Michael (2011), coconut water is a substance that improves callus induction, due content of high levels of Z-type cytokinin, it promotes cell division and rapid growth.

According to Manzoor *et al.* (2019), if the plant is polyploid, number of chromosomes, the cell size, and the nucleus size of that plant are higher than a normal plant cell. Therefore, by measuring these parameters, it can be determined that the plant is polyploid. Colchicine is an antimitotic agent that inhibits mitosis by preventing the polymerisation of tubulin, thereby disturbing the processes dependent on microtubule function such as cell motility, intracellular movement, cell polarity, and mitosis (Kamath *et al.*, 2008). Therefore, the most accurate way to detect the polyploidisation of the cell/plant is to count the chromosome numbers (Islam *et al.*, 2022). This results in doubling or changing the number of chromosomes thereby increasing the size of the cell's nucleus. However, counting of chromosomes is very difficult because the only phase of chromosome visualisation is in the metaphase of the mitosis cell division.

Conclusion

MS medium supplemented with 100 mL of coconut water was most effective in inducing the callus in turmeric.

The average nucleus area and perimeter of the treatment in 0.05% colchicine concentration showed the highest results. Therefore, 0.05% colchicine concentration found to be suitable for polyploid development in turmeric.

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