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Original Research Paper

Studies on the effect of induced polyploidy on yield and phytochemical content in *Centella asiatica* (L.) Urban

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ABSTRACT

Centella asiatica (L.) Urban, family Apiaceae, is a high value medicinal herb cum neutraceutical vegetable. The present investigation has successfully induced tetraploidy in the diploid *Centella asiatica* variety Arka Prabhavi thereby improving its biomass yield and secondary metabolite yield per unit area. The tetraploid was obtained by treating the shoot tips of diploid genotype (Arka Prabhavi) using 0.05% colchicine via cotton plug method. Tetraploid plants regenerated after six months showed significant changes in morphological traits like stolon length, intermodal length, leaf length, leaf width, petiole length and biomass yield. Tetraploidy was further confirmed with the cytological analysis showing doubling of the number of chromosomes and from the increased stomatal size, decreased stomatal density and increased size of pollen grains. Tetraploid and diploid genotypes were found to be on par for the asiaticoside content (2.37% and 2.30%, respectively) but since the biomass yield was increased (67%), the tetraploid reported 71.7% increase (91.09 kg/ha) in asiaticoside yield over the diploid Arka Prabhavi (53.05 kg/ha). Increase in asiaticoside yield will be beneficial for the pharmaceutical companies and herbal extractors who are interested in higher yield of metabolites in the extract. Analysis of the micronutrient (vitamin and minerals) content in the diploid and teraploid genotype showed significant increase in the tetraploid over the diploid genotype inferring that polyploidization has improved the nutrient levels also making it a promising green leafy vegetable.

Keywords: Arka Prabhavi, *Centella asiatica*, colchicine, tetraploidy, triterpenoid

INTRODUCTION

Centella asiatica (L.) Urban belonging to the family Apiaceae is a high value medicinal herb which is used both in the traditional and modern systems of medicine. The therapeutic uses involve memory enhancement, wound healing, radioprotective, antioxidative, antirheumatoic etc. The herb is valued for its leaves containing the triterpenoids namely asiaticoside, madecassoside, asiatic acid and madecassic acid. Besides medicinal uses, it is also consumed as a neutraceutical vegetable in many countries like India, Malaysia, China, and other parts of Asia. The herb is in high demand and massively collected from the wild for local use and export gradually depleting the natural population.

Polyploidy has found to play a significant role towards improving the morphological traits and biosynthesis of active metabolites in the medicinally important plants. Differences in the quantity and spectrum of active substances between diploids and polyploids have been reported in several medicinal plants species like *Datura stramonium* (Berkov & Philipov, 2002), *Scutellaria baicalensis* (Gao et al., 2002), *Spathiphyllum wallisii* (Eeckhaut et al., 2004), *Vitis vinifera* (Kara, 2022), *Platanus acerifolia* (Liu et al., 2007), *Eucalyptus globulus* (Liu et al., 2009) *and Papaver somniferum* (Mishra et al., 2010), etc. *C. asiatica* showed differences in the terpenoid content depending on the leaf morphology of the plants (Rohini & Smitha, 2022), and its ploidy level. Madagascar is the leading producer of this herb and the natural population in Madagascar comprises of both diploid and tetraploid types. The tetraploid plants were found to have more terpenoid content than diploid types (Rodier-Goud et al., 2013). With this background, an attempt has been made to induce polyploidy in the existing diploid variety of *Centella asiatica* (Arka Prabhavi) to improve it for the metabolite yield. Arka Prabhavi is a variety of *C. asiatica* with high content of asiaticoside but characterized by small leaves and spreading habit which makes it highly laborious for

the farmers to harvest, hence a need was felt to develop variety with high content and at the same time which grows tall and erect so that it becomes easy and economical for harvesting. Thus, the present investigation was taken up to induce polyploidy for improving the existing variety with respect to morphological traits and yield.

MATERIALS AND METHODS

Plant material and induction of polyploidy

The experiment was conducted using *Centella asiatica* variety Arka Prabhavi, which was used to produce the autopolyploid population with an intention to increase the biomass yield and asiaticoside yield. Two weeks grown seedlings were used for colchicine treatment to induce polyploidy. The experiment was conducted with five treatments of colchicine *viz.*, 0.01, 0.02, 0.03, 0.04 and 0.05% using completely randomized design with 3 replications and 15 seedlings per replication. Colchicine treatment was done on the shoot tips using cotton plug method. Cotton balls saturated with different concentrations of colchicine were placed on the seedling shoot tips and covered with aluminium foil for 24 hours to reduce the evaporation rate of the solution. After treatment, the running shoots were thoroughly washed with distilled water to remove traces of the colchicine solution. As soon as the nodal region proximal to the treated shoot tip produced new shoots it was excised from the mother plant and shifted to polybags with soil and cocopeat, maintained in the same greenhouse. Morphological variation with respect to leaf size was taken as the selection criteria for the putative polyploid genotypes. After proper root establishment, the plants were transplanted to the main field with the recommended package of practice (Farooqi & Sreeramu, 2004). The putative polyploids were examined six months later for cytology, stomatal and pollen size study to validate the polyploidy.

Cytological study

For cytological studies, cuttings of genotypes were raised in polybags to get fresh rooting and root tips were collected in the morning (10:00-10:15 am) for somatic chromosome studies. Fresh root tips were pre-treated with 0.003 M 8 hydroxyquinoline for two hours at 14-16°C followed by washing with distilled water and fixed in Carnoys fixative (Absolute alcohol: Glacial acetic acid: Chloroform in 6:3:1 ratio) for 24 hours. The next day, the root tips were rinsed in distilled water and then hydrolyzed by immersing in 5 N HCl for 30 minutes at room temperature. Hydrolyzed root tips were then transferred to Schiff's reagent after rinsing in distilled water and stored in dark for 2 hours. After 2 hours, the tips will be stained and then the stained tips are squashed with a drop of 1% aceto-carmine or orcein stain and observed under the microscope.

Palynology and stomatal studies

For palynological observations, pollen was collected from the diploid and polyploid genotype in the morning hours (9:00 am-10:00 am) from the freshly opened flowers. Flowers were lightly tapped with the needle so as to release the pollen followed by staining with 1% acetocarmine for observing under the microscope. Ten microscopic fields per genotype were observed for measuring the size of the pollen grains (pollen length and width).

For stomatal studies, five fully matured leaves were taken from diploid and polyploid genotypes. A thin nail polish film was applied on the lower surface of the leaf and after drying the epidermal layer was peeled off carefully (Hamill et al., 1992), placed on a slide and stained with safranin and viewed under the microscope to observe the stomatal characters like stomatal length, breadth, number of stomata/unit area and stomatal index (number of stomatal cells/total number of epidermal cells). Ten microscopic fields per genotype were observed for measuring each of the stomatal parameters.

Estimation of quantitative traits and biomass

Field evaluation of economically important quantitative traits like leaf length and breadth (cm), stolon length (cm), petiole length (cm), intermodal length (cm), number of nodes, number of leaves per plant and biomass yield was carried out in replicated block design with five genotypes (polyploid, Arka Prabhavi, Arka Divya, IIHR-CA-17 and IIHR-CA-18) and four replications for four seasons (March, 2022, July 2022, March 2023 and July 2023) over two consecutive years.

Phytochemical analysis for estimation of terpenoids

All the genotypes were subjected to phytochemical analysis using HPLC technique for analyzing the

active ingredients like asiaticoside, asiatic acid, madecassoside and madecassic acid by using the procedure standardized for *Centella asiatica* (Rohini & Smitha, 2022). The content was also evaluated for two consecutive years over four seasons (March 2022, July 2022 and March 2023, July 2023) to confirm the content stability of the tetraploid.

Vitamin and mineral analysis

Nutrient content of the polyploid (IIHR-CA-28) and diploid (Arka Prabhavi) was analyzed with respect to total carotenoids, Vitamin C and major and minor minerals in completely randomized design with 7 replications per genotype. Vitamin C content was estimated by 2, 6-dichlorophenol indophenol (DCPIP) titration method described by Rao & Deshpande (2006).

Estimation of total carotenoids

Five grams of the plant tissue was extracted in mortar and pestle with acetone and the acetone extract was made up to 100 ml in the volumetric flask. Flask was well shaken and filtered to remove any particulates present. Clear extract was taken for the spectrophotometer reading. O.D. of the clear extract was read at 470, 644.8 and 661.6 nm with acetone as blank solution. Total carotenoids was calculated as per the formula given here.

Ca= 11.24* A (661.6) - 2.14* A (644.8)

Cb= 20.13* A (644.8) - 4.19 * A (661.6)

Ca+ b = 7.05* A (661.6) + 18.09* A (644.8)

Cx+ C = 1000* A (470) – 1.90 Ca – 63.14Cb/214

Ca= Concentration of Chlorophyll a

Cb= Concentration of Chlorophyll b

Ca+ b = Concentration of Chlorophyll a+b

Cx+ C = Concentration of total carotenoids (Xanthophyll+ Carotenes)

Estimation of minerals

The collected plant samples were washed with distilled water to remove dusts and air dried. Then plant samples were dried at 60°C in a hot air oven for 24 hours and were powdered and stored for laboratory analysis. Nitrogen content in the biomass was estimated following the procedure described by Kjeldahl (1883) using Pelicans KEL PLUS System. Plant samples were digested with di-acid [10:4- nitric acid (HNO_3): perchloric acid ($HClO_4$)] mixture and mineral contents were extracted with 6 N hydrochloric acid (HCl) for analysis (AOAC, 1990). Phosphorus in plant samples were estimated by yellow colour method and sulphur by turbidimetric method using spectrophotometer (Model: Elico-minispec). Potassium was estimated using flame photometer (Model: Elico-CL378). Calcium, magnesium and micronutrients (Fe, Cu, Zn, and Mn) in di-acid extracts were analyzed using atomic absorption spectrophotometer (Model: Analyst- E200, Perkin Elmer).

Statistical analysis

The statistical differences among means of all data of diploid and autopolyploid plants were computed by analysis of variance (ANOVA) with a Tukey's test by using grapes Agri 1. Software (Gopinath et al. 2021).

RESULTS AND DISCUSSION

Induction of polyploidy

The genotypes showed survival up to 0.05% colchicine treatment above which the survival rate was extremely less and subsequently all treated plants dried up. There was a significant difference between the survival rates based on the colchicine concentration used. Survival percentage of treated plants ranged from 13 to 40% with least survival for 0.05% treated plants (13%). But among the survived plants, morphological variation was observed only for plants treated with 0.05% colchicine (Table 1). Hence, those surviving plants were multiplied and the population was raised for further confirmatory studies along with the diploid genotypes. The results of this experimental study showed that the percentage of survival was dependent on the concentration of the colchicine used. The efficiency and success rate of polyploidy induction in plants is low due to factors such as undesirable chromosomal changes, the creation of chimeric plants, and a lack of root production or even the death of treated plants (Madani et al., 2021). Significant morphological variation in leaf size was observed for the seedlings treated with 0.05% colchicine treatment and was found effective in the production of tetraploid progeny. Only two out of the fifteen plants survived after the 0.05% colchicine treatment showing that colchicine concentration had a direct effect on the plant physiology and survival rate.

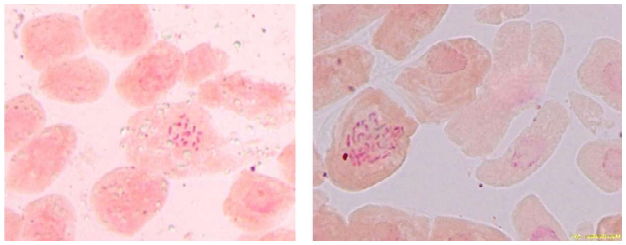
Table 1 : Survival percentage of *Centella asiatica* plants obtained after the colchicine treatment

Colchicine (%)	Plants treated (Nos.)	Survived plants (%)	Morphological variation
0.01	15	39.99[a1]	No
0.02	15	38.33[a]	No
0.03	15	33.33[a]	No
0.04	15	29.99[ab]	No
0.05	15	13.33[b]	Yes

[1]within each column values followed by different letters are significantly different (P = 0.05), by Tukey test

Cytological study

Mitotic analysis of both the genotypes showed that the somatic cells of diploid plants had 2n=2x=18 chromosomes, while, the polyploid genotypes had double the number of chromosomes 2n=4x=36 showing tetraploidy (Fig. 1). As stated by Williams & Oliveira (2020), polyploidy has cell-level phenotypic consequences arising from increased bulk DNA amount and numbers of genes and their interactions.



Diploid, 2n=18 (Arka Prabhavi) | Tetraploid, 2n=36 (IIHR-CA-28)

Fig. 1 : Metaphase stage of mitosis in diploid and tetraploid genotypes

Palynology and stomatal studies

The increase in cell sizes as a result of chromosome doubling was also evident from the studies on pollen grains which showed that there is a significant increase in the size of pollen grains in the tetraploid genotypes when compared to the diploid counterpart (Table 2 & Fig. 2). Increase in pollen length (37%) and breadth (10%) showed a significant increase in size in the tetraploid as compared to the diploid genotype. Similarly, stomatal studies showed that the length and breadth of stomata increased in the tetraploid as compared to the diploid but the number of stomatal openings per unit leaf area decreased in the tetraploid that may be accounted because of increase in stomatal size and therefore the stomatal index also got reduced (Table 2 and Fig. 2). Pollen grains size also provides a quick indicator to validate polyploidy in plants (Blakeslee, 1941). The present study has also reported an increased size of pollen grains in the tetraploid genotype which has also been observed by other workers like Omid et al. (2010) in *Ocimum basilicum* and Srivastava (2002) in *Helianthus annuus* L. Use of stomatal studies for confirming polyploidy has been

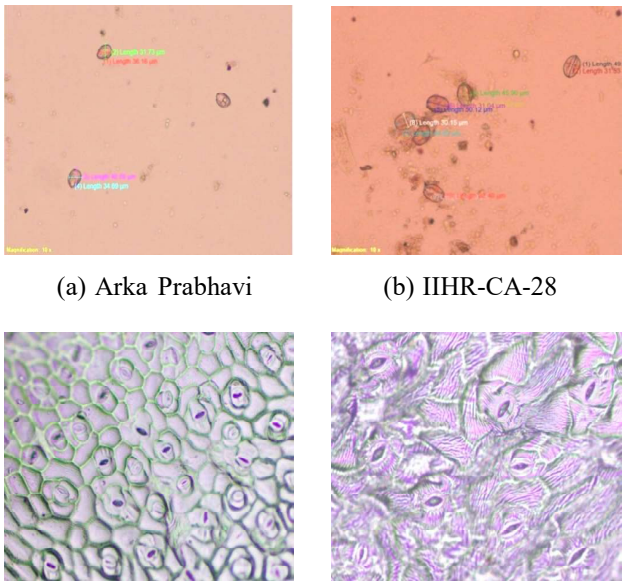
Table 2 : Comparison of pollen size and stomatal characters in the tetraploid and diploid genotypes of *C. asiatica*

Trait	Tetraploid (IIHR-CA-28)	Diploid (Arka Prabhavi)	CV	SE(m)
Pollen length (µm)	48.38[a1]	35.19[b]	6.34	1.002
Pollen width (µm)	33.29[a]	30.21[b]	8.26	0.991
Stomatal length (µm)	12.30[a]	10.36[b]	3.57	0.153
Stomatal width (µm)	8.32[a]	5.51[b]	3.68	0.096
No. of stomata/unit area	10.00[b]	28.57[a]	7.88	0.574
Stomatal index (%)	23.07[b]	55.71[a]	10.27	1.53

[1]Within each column values followed by different letters are significantly different (P = 0.05), by Tukey test.

*CV: coefficient of variation; SE: standard error of means

already attempted in *C. asiatica* by Kaensaksiri et al. (2011) where they found that tetraploid plants demonstrated significantly longer stomata and a higher stomatal index compared to those of the diploid control plants, the same result has been reproduced in this study as well.



(a) Arka Prabhavi (b) IIHR-CA-28

(c) Arka Prabhavi (d) IIHR-CA-28

Fig. 2 : Pollen grains as observed in (a) diploid, Arka Prabhavi (b) tetraploid, IIHR-CA-28 and stomata as observed in (c) diploid, Arka Prabhavi (d) tetraploid, IIHR-CA-28

Evaluation of quantitative traits and biomass

Comparison of quantitative traits between diploid and tetraploid *C. asiatica* revealed that phenotype of the tetraploid was different from that of the parental diploid plants (Fig. 3). There was a significant difference between the tetraploid and diploid genotypes with respect to quantitative traits like stolon length, leaf length and breadth, petiole length, internode length, number of leaves and number of nodes (Table 3). On the other hand, traits like number of nodes/plant and number of leaves did not show significant difference between the genotypes. In all the harvests, tetraploid IIHR-CA-28 recorded significantly higher fresh and dry biomass yield over the dipliod check variety Arka Prabhavi. Pooled analysis of all seasons showed that IIHR-CA-28 recorded 53.7% increase in fresh yield (28.064 t/ha) over Arka Prabhavi (18.259 t/ha) and 67% increase in dry yield (3.848 t/ha) over Arka Prabhavi (2.303 t/ha) (Table 4). The significant improvement in the traits like stolon length, leaf length, leaf breadth, petiole length, intermodal length and biomass yield in the tetraploid over the diploid plants which are in line with the findings of Doyle & Coate (2019) stating that significant effects of ploidy were noted on plant growth and morphology because of the increased size of somatic cells and increased content of cell sap leading to the expansion of vegetative parts.

Phytochemical analysis

The ploidy level of plants is associated with morphological and biochemical characteristics and its modification have been used as a strategy to alter the quantitative and qualitative patterns of secondary metabolite production in different medicinal plants (Madani et al., 2021). Phytochemical analysis of the

Table 3 : Quantitative trait comparisons of tetraploid and diploid genotypes of *C. asiatica* over 2 years

Genotype	Stolon length (cm)	No. of leaves	Petiole length (cm)	Leaf length (cm)	Leaf breadth (cm)	Internode length (cm)	No. of nodes
IIHR-CA-28 (tetraploid)	91.85^{a}	18.95	31.25^{a}	4.80a	7.50^{a}	13.18^{a}	7.35
Arka Prabhavi (check)	54.42^{d}	17.70	18.95^{c}	3.06^{c}	4.41^{c}	7.91^{b}	7.15
Arka Divya	85.55^{ab}	20.10	29.40^{a}	4.80^{a}	8.025^{a}	12.67^{a}	7.7
IIHR-CA-17	73.35^{bc}	17.80	23.52^{b}	3.84^{b}	6.165^{b}	11.24^{a}	7.25
IIHR-CA-18	70.60^{c}	19.15	23.12^{bc}	3.83^{b}	6.015^{b}	11.49^{a}	7.5
CD at 0.05	12.257	NS	4.252	0.544	1.029	3.236	NS
CV (%)	10.585	7.679	10.928	8.67	10.396	18.584	15.288

Significant difference between treatments indicated by different alphabets (P = 0.05)

Table 4 : Analysis of tetraploid with check variety (diploid) and other lines diploids for biomass yield over different seasons

Genotype	FBY* (t/ha)	DBY* (t/ha)	FBY* (t/ha)	DBY* (t/ha)	FBY* (t/ha)	DBY* (t/ha)	FBY* (t/ha)	DBY* (t/ha)	FBY* (t/ha)	DBY* (t/ha)
	March, 2022		July, 2022		March, 2023		July, 2023		Pooled	
IIHR-CA-28 (Tetraploid)	22.346^{a}	4.469^{a}	36.883^{a}	3.939^{a}	24.660^{a}	3.300^{a}	32.625^{a}	3.683^{a}	28.064^{a}	3.848^{a}
Arka Prabhavi (check)	13.038^{d}	2.608^{d}	17.998^{d}	2.212^{c}	16.208^{cd}	2.098^{c}	21.895^{c}	2.293^{d}	18.259^{c}	2.303^{d}
Arka Divya	23.168^{a}	4.634^{a}	26.703^{b}	3.391^{ab}	22.783^{ab}	2.938^{ab}	29.590^{ab}	3.143^{b}	26.282^{a}	3.527^{b}
IIHR-CA-17	18.559^{b}	3.712^{b}	23.785^{bc}	3.006^{b}	19.733^{bc}	2.483^{bc}	26.073^{bc}	2.851^{bc}	22.609^{b}	3.013^{c}
IIHR-CA-18	15.434^{c}	3.087^{c}	20.023^{cd}	2.644^{bc}	13.483^{d}	2.040^{c}	16.493^{d}	2.433^{cd}	15.476^{d}	2.551^{d}
CD at 0.05	2.05	0.41	5.026	0.772	3.665	0.558	4.463	0.45	7.441	0.296
CV (%)	6.854	7.187	13.008	16.499	12.277	14.087	11.434	10.143	2.538	6.3

*FBY: fresh biomass yield; DBY: dry biomass yield; significant difference between treatments indicated by different alphabets (P=0.05)

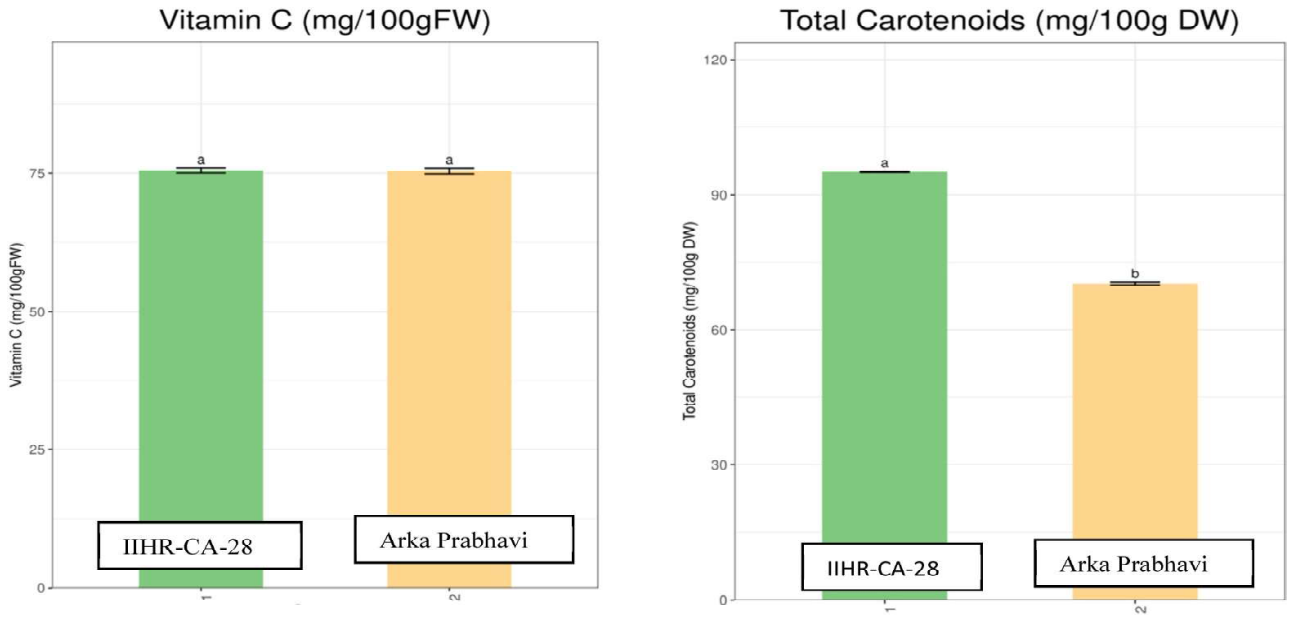
IIHR-CA-28 (tetraploid) Arka Prabhavi (diploid)

Fig. 3 : Morphological comparison of tetraploid (IIHR-CA-28) and diploid (Arka Prabhavi)

genotypes for active ingredient like asiaticoside, madecassoside, asiatic acid and madecassic acid contents using HPLC method revealed that the tetraploid and diploid accumulated the same quantity of metabolites irrespective of the ploidy level. The evaluation for active ingredient content showed that the tetraploid, IIHR-CA-28 was either superior or on par with the diploid check variety Arka Prabhavi for the active metabolite content over the different harvest season. Pooled data showed that IIHR-CA-28 (2.37%, 5.39%) was found to be on par with Arka Prabhavi (2.30%, 5.32%) for the asiaticoside and total terpenoid content (Table 5). Asiaticoside, which is the most important terpenoid in leaves of *C. asiatica* was observed to be 2.37% in the tetraploid whereas in the diploid it was 2.30%. But, in terms of asiaticoside yield (kg/ha), the tetraploid reported 71.7% increase (91.09 kg/ha) over the diploid Arka Prabhavi (53.05 kg/ha). Thus, the induction of polyploidy has significantly increased the yield of metabolites in tetraploid because of increase in its vegetative

Table 5 : Comparative values of active metabolites in the tetraploid and diploid genotypes of *C. asiatica* (pooled data of 2 years)

Genotype	Madecassocide (%)	Asiaticoside (%)	Madecassic acid (%)	Asiatic acid (%)	Total triterpene content (%)	Asiaticoside yield (kg/ha)
IIHR-CA-28 (tetraploid)	2.33	2.37	0.11	0.14	5.39	91.09
Arka Prabhavi (check)	2.17	2.30	0.20	0.17	5.32	53.05
Arka Divya	1.47	1.15	0.10	0.10	3.06	40.66
IIHR-CA-17	1.68	1.30	0.08	0.11	3.44	39.21
IIHR-CA-18	1.81	1.80	0.14	0.15	4.20	45.81
SD	0.354	0.557	0.044	0.029	1.060	21.451
SE(m)	0.158	0.249	0.020	0.013	0.474	9.593



Means followed by the same letters are not significantly different according to Tukey's test (P =0.05)
Bars represent means of seven replicates ± SE

Fig. 4 : Graph showing the comparative values of Vitamin C and total carotenoids in the diploid (Arka Prabhavi) and tetraploid genotype (IIHR-CA-28) of *Centella asiatica*

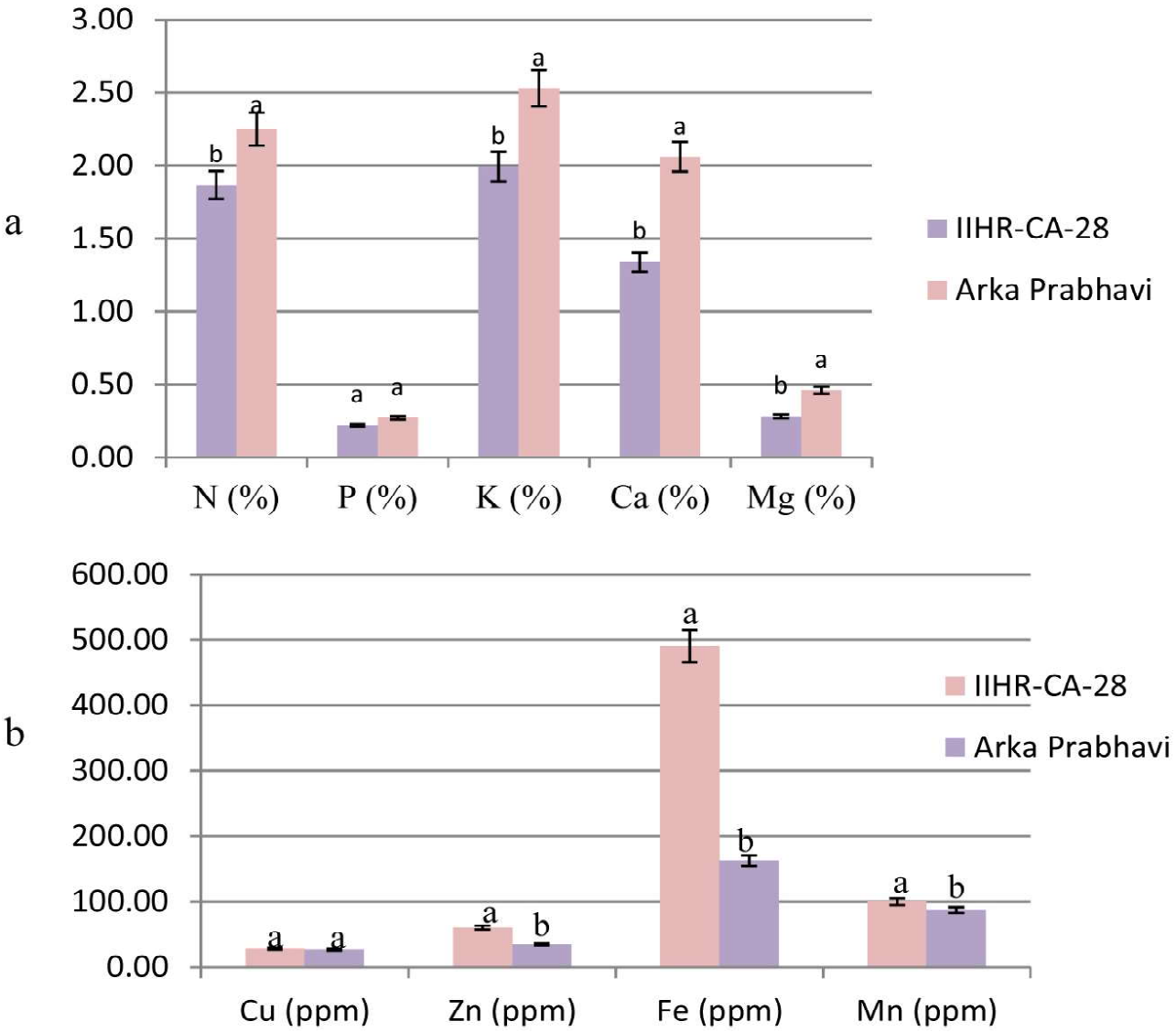
biomass. In most of the medicinal plants, the polyploids have reported a higher content of active metabolites compared to their diploid counterparts (De-Jesus & Weathers, 2003), but in the present study, the content of the active metabolites has not been altered by the ploidy induction and hence the metabolite content is equal in both the tetraploid and diploid genotypes. Similar observation was found in hop cones where the contents of major chemical constituents was little affected by ploidy level and also the total essential oils were significantly lower than those in diploids (Trojak & Skomra, 2013). But nevertheless, the increase in biomass yield has significantly increased the yield of the triterpenoids obtained per unit area in the induced tetraploids which will be an additional benefit for the farmers and industries cultivating and exploiting this herb.

Vitamin C, total carotenoid and mineral analysis

Additional use of the herb as a green leafy vegetable impelled us to carry out the nutritional analysis with respect to the micronutrients like total carotenoids, vitamin C and major and minor minerals. The analysis for Vitamin C content showed that both the tetraploid (75.47 mg/100 g FW) and diploid genotypes (75.36 mg/100 g FW) were on par, whereas, the total carotenoid content was found to be high in the tetraploid genotype IIHR-CA-28 (95.07mg/100 g dry weight) as compared to the diploid genotype (70.31 mg/100 g dry weight) (Fig. 4).

The tetraploid and diploid genotypes were subjected to mineral analysis for estimating the quantity of major and minor mineral elements present in them. In this study, it was observed that the diploid genotype was found superior for all the major minerals like nitrogen, potassium, calcium and magnesium whereas the tetraploid genotype was found superior for most of the minor minerals like zinc, iron and manganese (Fig. 5). The genotypes were found on par for the phosphorous and copper content.

Comparison of the micronutrients between the diploid and autopolyploid is first of its kind attempted in this crop. A significant increase in the total carotenoid and micro mineral content of tetraploid over the diploid genotype also emphasizes the fact that autopolyploids are characterized by genome duplication leading to the increased activity of previously weakly expressed genes in a target biosynthetic pathway and improving the metabolite production. This may be explained due to reduction in the ratio of the membrane to the amount of chromatin, which leads to increased contact between the chromatin material and the nuclear membrane and enhanced activity of the gene for each cell.



Means followed by the same letters are not significantly different according to Tukey's test (P =0.05)
Bars represent means of seven replicates ± SE

Fig. 5 : Comparative data of major (a) and minor (b) minerals in diploid and tetraploid genotype of *Centella asiatica*

CONCLUSION

Increasing the plant biomass yield and active metabolite content is currently the biggest challenge for the medicinal plant breeders. Through polyploidy breeding, the present investigation has successfully induced tetraploidy in the diploid *Centella asiatica* variety Arka Prabhavi thereby improving its biomass yield and secondary metabolite yield per unit area.

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