ISSN (Online): 2349-1183; ISSN (Print): 2349-9265
TROPICAL PLANT RESEARCH
The Journal of the Society for Tropical Plant Research

8(3): 210–216, 2021
DOI: 10.22271/tpr.2021.v8.i3.026

Research article

In vitro cytological studies of *Passiflora vitifolia* Kunth an important medicinal and ornamental plant

K. P. Chandana[1], N. K. Hemanth Kumar*[1,2], H. S. Prithviraj[1,3] and Shobha Jagannath[1]

[1]Plant Tissue Culture Laboratory, Department of Studies in Botany, University of Mysore, Manasagangothri, Mysore, Karnataka, India
[2]Department of Botany, Yuvarajas College, University of Mysore, Mysuru, Karnataka, India
[3]Department of Botany, Sri Mahadeshwara Government First Grade College, Kollegal, Karnataka, India

*Corresponding Author: hemanthbot@gmail.com
[Accepted: 19 December 2021]

Abstract: The study was carried out to determine the *in vitro* cytological behaviour of callus cultures of *Passiflora vitifolia* member of Passifloraceae. The callus cultures were raised using stem and leaf explants on MS medium supplemented with different growth regulators of axuin (2,4-D, IAA and NAA) and cytokinin (BAP and Kn.) and the combination of auxins with cytokinin (NAA + BAP) and (NAA + Kn.). Best callusing was achieved in the leaf and stem explants in NAA and 2-4,D at 1.5 mg l^{-1}. Cytogenetic analysis of the callus was carried out for primary callus culture as well as 4-month-old callus culture. The chromosome number showed wide range of variation, however, the majority of the cells were observed to be diploid (2n = 18) in nature. The most observed cell and chromosomal irregularities were polyploid cells, enucleated cell, binucleatedcell, chromosomal bridges were found to be more common. The composition and concentrations of media were found to effect on chromosomal instability and in turn, these observations showed that genetic variations may arise during callogenesis.
Keywords: Passiflora - Cytology - Callus culture - Diploid cells.

[Cite as: Chandana KP, Hemanth Kumar NK, Prithviraj HS & Jagannath S (2021) *In vitro* cytological studies of *Passiflora vitifolia* Kunth an important medicinal and ornamental plant. *Tropical Plant Research* 8(3): 210–216]

INTRODUCTION

The discipline of plant tissue culture has been an area of investigation during the past few decades and it has found applications in conventional breeding methods used for the improvement of crops. Genetic variability is the key factor in any breeding method. The genetic variability created through conventional breeding techniques is slow and dependent on recombination (Mascarenhas 1991).

The stability of *in vitro* culture is a major problem in applying plant cell and tissue culture techniques to basic and applied research. The variability of chromosome can also be a source of somaclonal variation. The explants source is considered to be one of the important factors. The factors involved in the stability or variability of *in vitro* cultures have been described in many detailed reviews (Sacristan 1971, Karp 1989). Chromosomal abnormalities are responsible for such variations under the influence of chemical and physical culture conditions *in vitro* (Bayliss 1980, Larkin & Scowcroft 1981, Mukhopadhyay *et al*. 2000).

Cytological studies provide pave the way to detect the somaclonal variations and reduced the stability of plants system (Raha & Roy 2003). Several studies have reported that chromosome number and structural changes occur from tissue culture and that chromosomal instability can be induced by media components, culture age, explant tissue and even by plant genotype. Knowledge of chromosome structure has played a crucial role in the improvement of medicinally important plant species and has far-reaching implications (Lee & Phillips 1988).

The results obtained from chromosomal studies might be also helpful in the field of taxonomy and relationship studies. On the other hand variation in chromosome structure and number disturbs the physiological and genetic balance of the callus leading to a loss in the capacity to regenerate plants (Singh 1986). Thus, regeneration of plants would to be linked with the chromosomal behaviour of the source, callus culture (Tha &

Received: 01 March 2021 Published: 31 December 2021
https://doi.org/10.22271/tpr.2021.v8.i3.026

Roy 1982). Therefore, there is a need to establish the nature and source of variation in cultured cells. Factors responsible for variation in cultured cells may beage of culture; source of explants, the genotype of the explants, incubation conditions during culture, concentration and type of growth regulators used in the media (Ramulu 1987). In the present investigation, the chromosomal variations in the callus culture of *Passiflora vitifolia* Kunth are a rare has been studied (Beninca *et al*. 2007).

MATERIALS AND METHODS

Collection and inoculation of plant materials

Young and healthy leaf and stem explants of *Passiflora vitifolia were* collected from Lalbagh garden, Bangalore. The explants were washed under running tap water to remove soil and dust particles followed by washed with fungicide bavistin (2%, w/v) for ten minutes followed by washing with sterile double distilled water. Finally, explants were washed with 0.01% mercuric chloride (w/v) for five minutes then washed with sterile double distilled water three times to ensure no traces of mercuric chloride are left. The explants were placed onto the sterile blotter discs to drain out the excess water and were cut into small pieces of size 1 cm^2. The sterilized and trimmed explants were inoculated onto the MS medium with 3% sucrose and gelled with 0.8% agar as a solidifying agent and supplemented with various concentrations of auxins (2,4–D, IAA, NAA), Cytokinin (BAP, Kn) and the combination of auxins with cytokinin (NAA + BAP) and (NAA + Kn). The pH of the medium was adjusted to 5.8 and autoclaved for 20 minutes at 121°C for 15 minutes. The inoculated cultures were maintained at 26±2°C with a photoperiod of 18 hours daylight and 6 hours dark. After 2 weeks of incubation, the calli were subjected to cytological studies. The proliferation of the same calli was continued and fixed at the end of 9^{th} week (Khan *et al*. 1988, Diallo *et al.* 2008).

Pretreatment of callus for cytological studies

The leaf and stem calli segments were pretreated with 0.02M8 Hydroxyquinoline for three and half hours. Then calli segments were washed thoroughly water and transferred to Carnoy`s- I fixative3:1 ratio (Absolute alcohol: Glacial acetic acid) for 24 hours. After 24 hrs calli segments were washed thoroughly withwater to remove traces of fixative and stored in 70% alcohol.

Staining and squashing

A segment of the callus was placed in a mordant (0.1% Ferrous ammonium sulphate solution) for 5 minutes. Excess mordant was drained off with 45% propionic acid and stained with propionohematoxylin stain for 10 minutes. Stained callus was kept in 0.1% propionic acid before squash preparation and the permanent slides were prepared in butanol : acetic acid ratio (1:1) and mounted in DPX followed by slides were incubated at 60°C overnight (Sudarshana *et al.* 2015).

RESULTS

Callus was initiated from the cut ends of the leaves and internode after fourth weeks of inoculation (Figs. 1A & 1B). Out of the three auxins used, NAA and 2-4D were found to be the best for callus induction and profuse callusing was observed at $1.5mgl^{-1}$. In cyokinin BAP (1.5 mg l^{-1}) and Kn (0.5 mg l^{-1}) showed best response. The NAA+BAP (Leaf 2.0 + 1.5 mg l^{-1} and stem 1.5 + 1.0 mg l^{-1}) and NAA+Kn (leaf 1.5 + 2.0 mg l^{-1} and stem 1.0 + 1.5 mg l^{-1}). The calli from all the above combinations were fixed to investigate cytological behaviour. The stem and leaf callus tissues consist of parenchyma, meristematic cells and treachery elements.

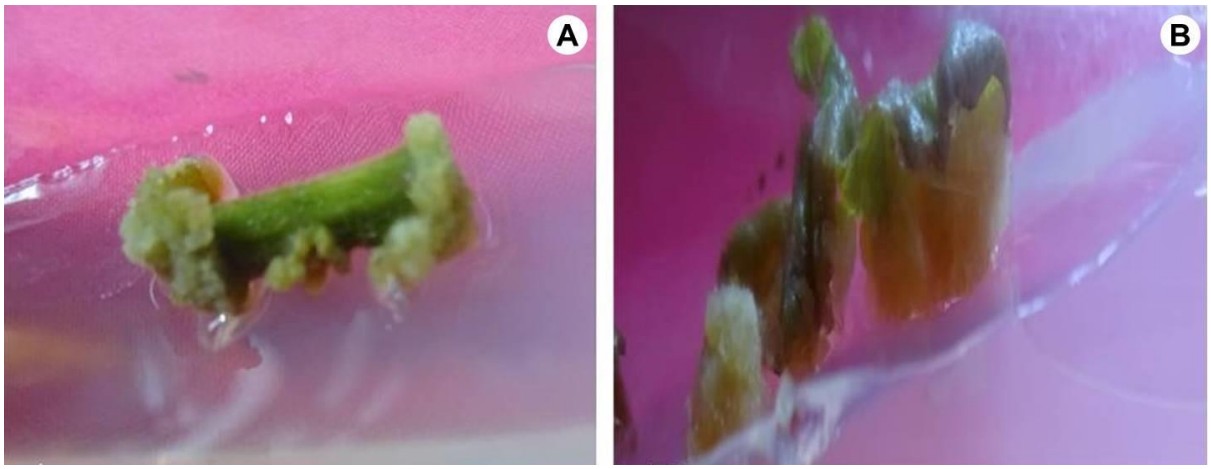

Figure 1. Callus proliferation from stem (**A**) and leaf (**B**).

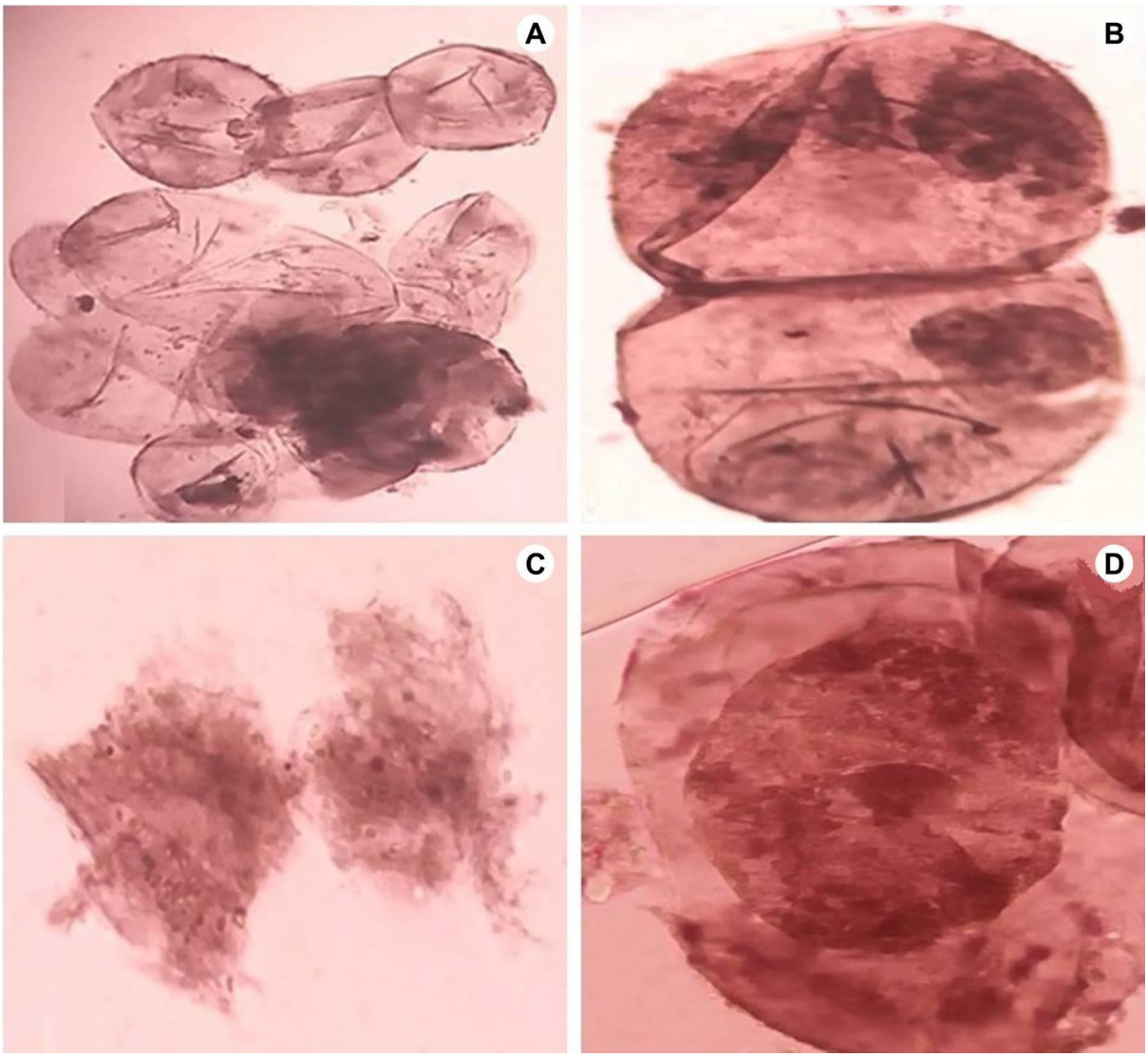

Figure 2. Cytological variation in leaf callus of *Passiflora vitifolia* Kunth: **A**, Enucleated cell; **B**, Binucleated cells after diffusion but not seprated nucleus of various shape; **C**, Polar view of Metaphase; **D**, Cell with in a cell.

One month of old callus was found to contain enucleated cells. In general, enucleated condition of the callus was due to the migration of the nuclear content from the cells through the papillae projections or it might be due to the disintegration of the nucleus (Fig. 2A). In two-month-old callus the chromosomes are more condensed and coiled. Metaphase is characterized by the arrangement of chromosomes at the equatorial plane of the cell before being separated into each of the two daughter cells (Fig. 3F). In both the explants a good percent of callus cells exhibited multinucleolated condition starting from uninucleolate to bi, tri, tetra and multinucleolate condition. The number of nucleoli in a multinucleolate differ within a single cell *i.e.*, 2 nucleoli (Fig. 2B), 3 nucleoli (Fig. 3A), 4 nucleoli in the trinucleated cell is a characteristic feature. The size of the nucleoli is not the same in each nucleus of a multi nucleolated cell. The shape of nucleoli varied from spherical to oval to column and sometimes lobed. In a binucleated cell, 4-nucleoli were found in each nucleus.

During early prophase, the chromosome becomes visible as they condense and become shorten, coil, and thicken. The callus cells of *Passiflora vitifolia* showed an important characteristic feature in that the content of the cytoplasm along with nucleus flowing out of the cell. In some cells extensions of the cells in the form of papillate projections were found in the cultured cells of *Passiflora vitifolia* and these projections may get separated from the cell wall &get separated due to the formation of new cell between them, resulting a new cell& the other cell becoming empty. In one of the cells, the content of the cell entered into another cell resulting in cytomixis and endoreduplication (Figs. 4C & 4D).

Xylem differentiation may serve as a model for studying other types of cytodifferentiation in plant tissues. Cytodifferentiation includes the deposition of a specifically patterned secondary cell wall, its subsequent lignification, then a loss of the nucleus, cell contents & death of the cell (Figs. 5C & 5D). We also observe the Polar view of Metaphase (Fig. 2C). Cell with in a cell (Fig. 2D), telophase (Fig. 3C), Late prophase (Fig. 3D), A

Polyploidy stage showing late prophase (Fig. 3E) in leaf callus. Variation in cell shape and sizes of cultured cells (Fig. 4A). The flowing of nuclear content from one nucleus to another nucleus within a single cell (Fig. 4B), Telophase and polar view of metaphase is also observed in stem callus.

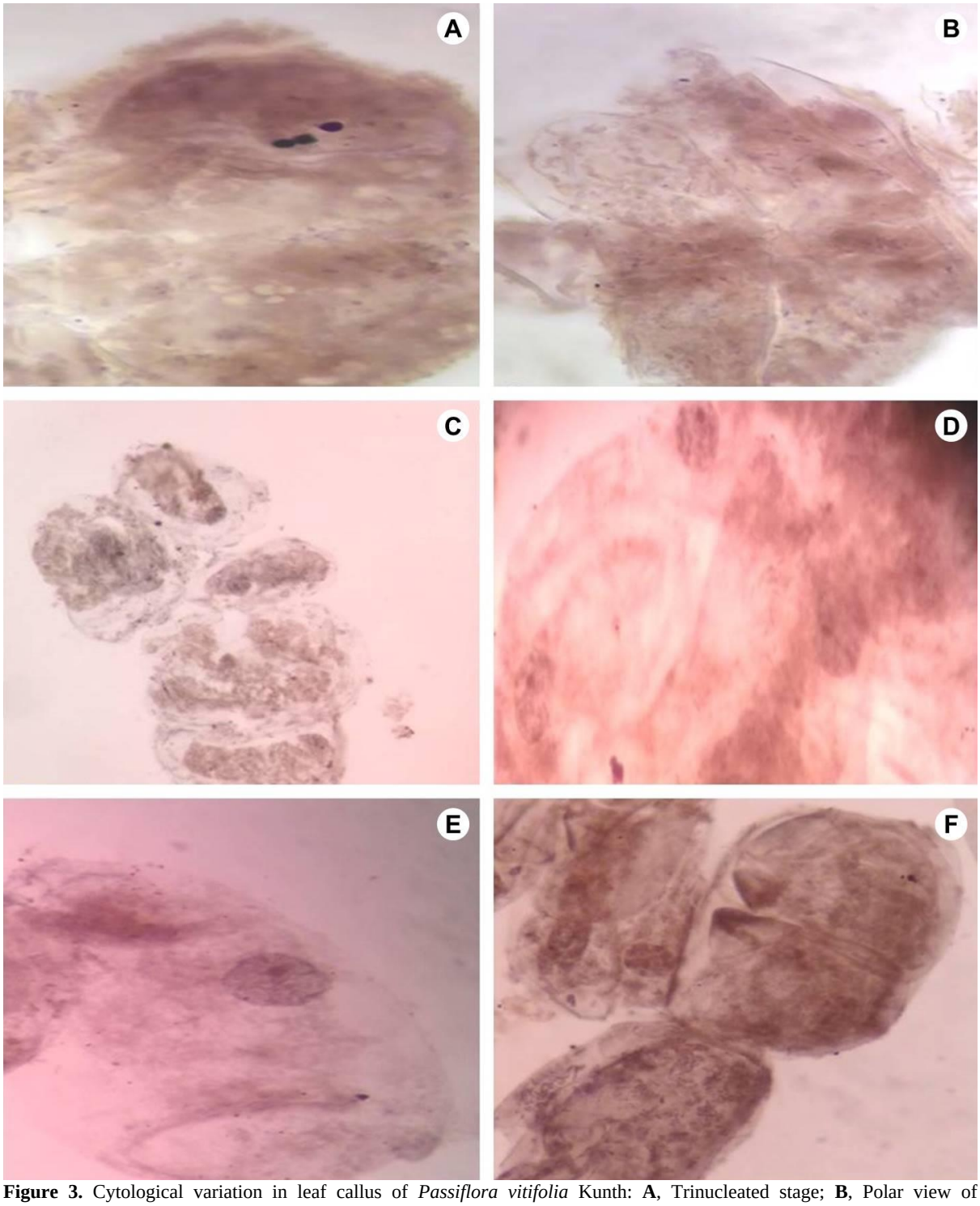

Figure 3. Cytological variation in leaf callus of *Passiflora vitifolia* Kunth: **A**, Trinucleated stage; **B**, Polar view of Metaphase; **C**, Telophase; **D**, Late prophase; **E**, A polyploidy Stage showing late prophase; **F**, Polyploidy cell showing Metaphase.

DISCUSSION

Callus induction was observed on both leaf and nodal segments on MS medium augmented with NAA. The degree of callus production varied with reference to the supplementation of the plant growth regulators in the medium. The Highest degree of callus induction was observed on MS medium augmented with 2-4D (1.0–1.5 mg l^{-1}) and NAA (1.5 mg l^{-1}) respectively. The efficacy of exogenous 2, 4-D found in this experiment, was also been reported with other medicinal plants by various authors. The earlier results of Rani *et al*. (2003), Thomas & Maseena (2006), Hassan *et al*. (2009), Davallo *et al*. (2014) are in accordance with the present findings by

providing growth regulator in the culture medium for callus induction of *Withania somnifera* L., *Cardiospermum halicacabum* L., *Abrus precatorious* L. and *Jasminum sambac* L. respectively.

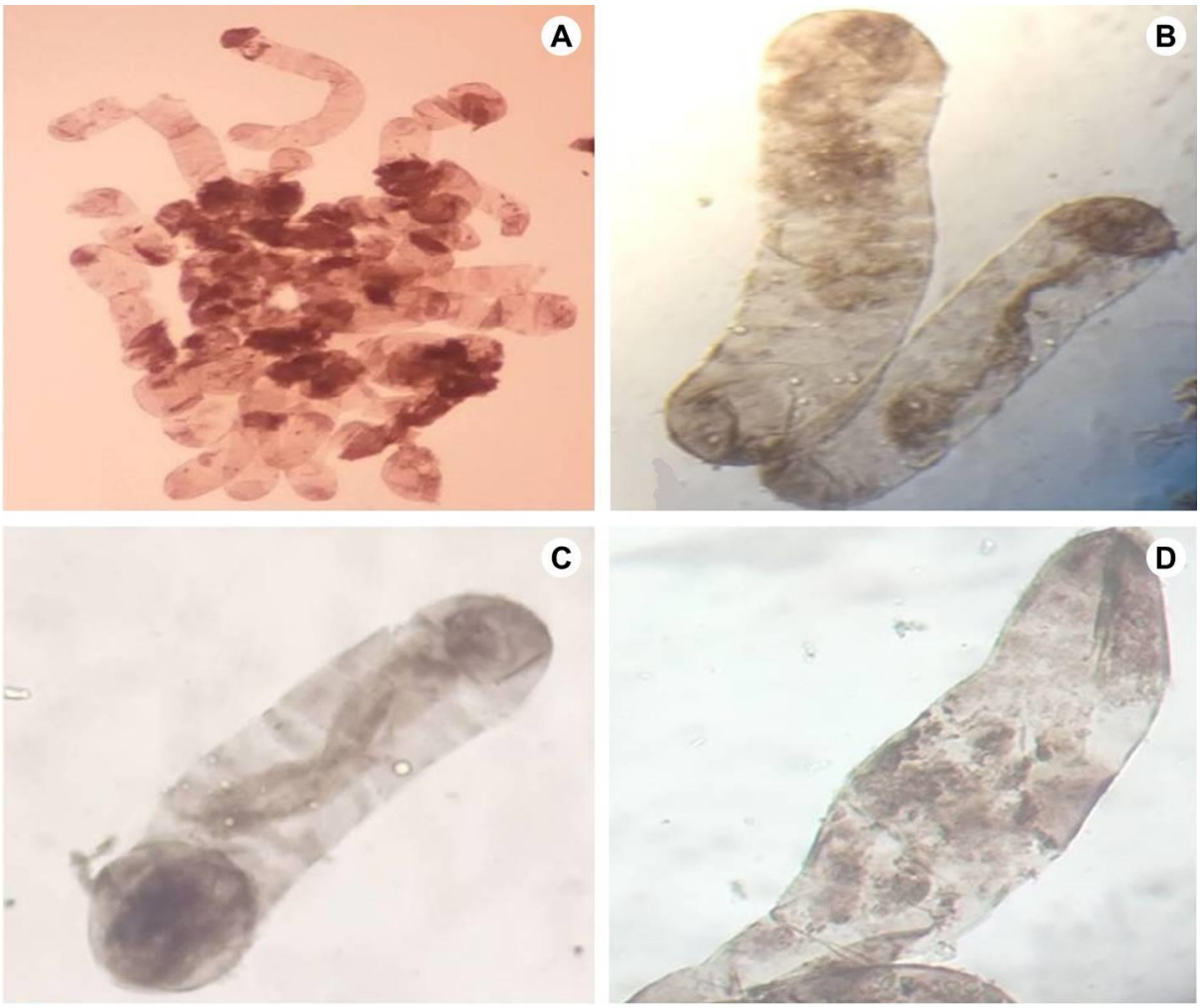

Figure 4. Cytological variation in stem callus of *Passiflora vitifolia* Kunth: **A**, Variation in cell shape and sizes of cultured cells; **B**, The flowing of nuclear content from one nucleus to another nucleus Within single cells; **C–D**, Elongated nucleus having spindle shaped nucleolus.

The cytological studies revealed that the cultured cells display heterogeneity both in chromosome complement and in nuclear changes like the structure of nuclei, asymmetric cell populations with polymorphic nuclei and different ploidy levels. The calli showed several types of cells exhibiting cytological variations including non-dividing cells with round nuclei, uninucleated cells, binucleated cell, enucleated cells, polyploidy cell, normal diploid cells, cells showing cytodifferentiation and cytokinesis. Our results are following the earlier studies carried on *Xanthophyllum flavascens* Roxb. (Sudarshana *et al*. 2015).

The presence of nuclei and nucleoli in a single cell suggested that dramatic metabolic changes. Endoreduplication, endomitosis and fusion of nuclei as the mechanisms of polyploidization in tissues of polysomatic plants cultured *in vitro*. The present investigation in comparison to the primary culture the frequency of polyploid cells was higher in 4 months old culture. A steady increase in the frequency of polyploid cells is higher than diploids with the increase in the concentration of plant growth regulators. Irregular cell division such as nuclear division without cell division is expected to occur in callus cells with nuclei multiplication (Amato 1986). Asynchronous mitosis in a binucleated cell and a cell with other than the euploidy number in somatic tissue is one way by which cell varying in chromosome number could be derived. Asynchronous mitosis in the multinucleate cell also would explain the variation in chromosome number (Heinz *et al*. 1971).

The polyploidcell was encountered more frequently and a decrease in the frequency of dividing cells with an increase in polyploidy cell was seen with increasing age of culture which can be seen in the 2 months old callus of *Passiflora vitifolia*. The results are in accordance with earlier study of Azam & Amal (1989) on *Trigonella foenum-graecum* L. Similarly, variously shaped nuclei were observed in the callus cells of *Passiflora vitifolia*.

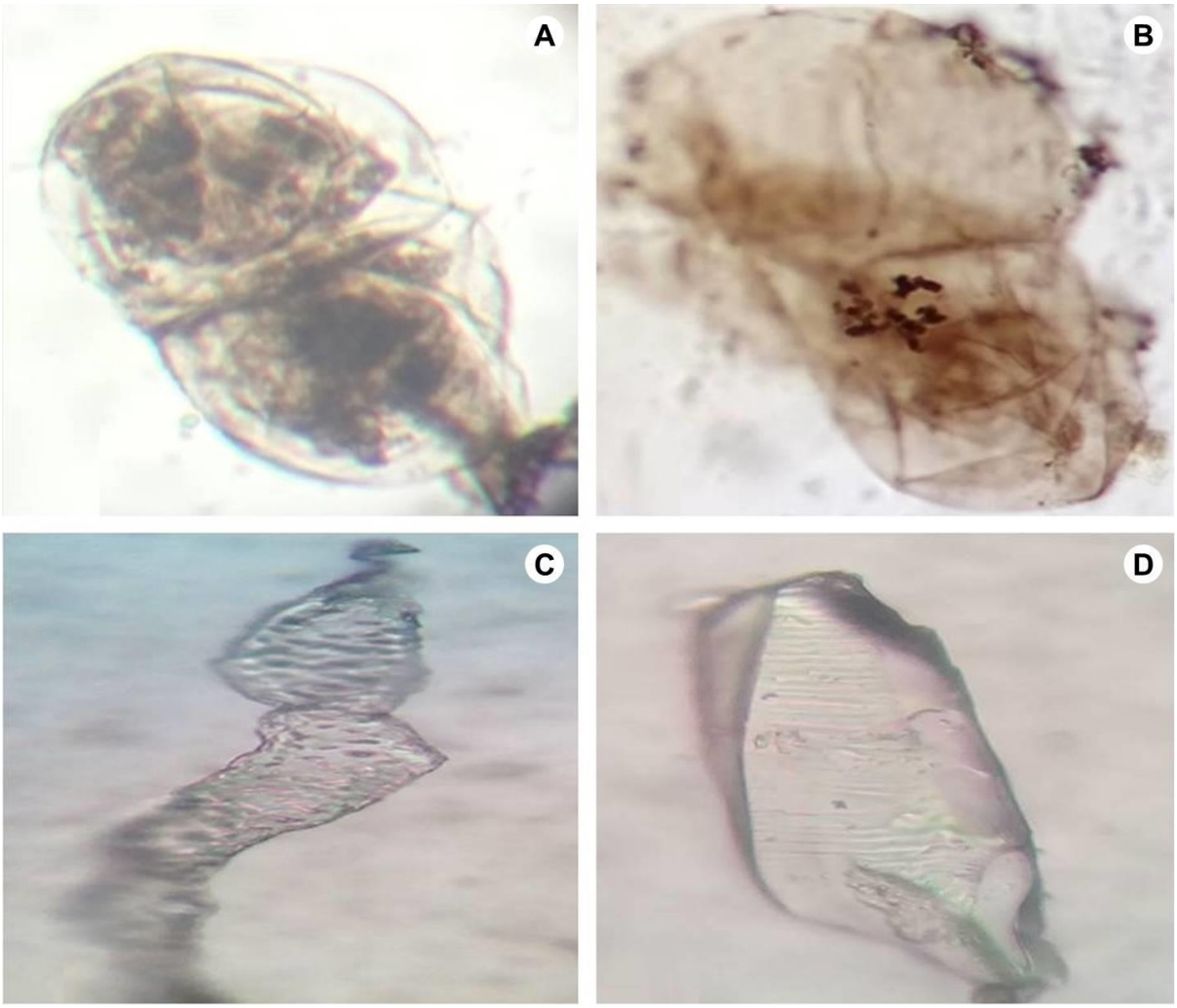

Figure 5. Cytological variations in stem callus of *Passiflora vitifolia* Kunth: **A**, Telophase; **B**, Polar view of Meta phase; **C**–**D**, Cytological differentiation (Xylem differentiation).

CONCLUSION

From the results of present study it can be concluded that the immature tissue of the plants contain maximum potentiality of dividing and they are more responsive for callus induction. The solid MS medium containing 1.5 mg l^{-1} NAA and 2-4, D shows the high percentage of callus initiation from young leaf and internode. Callus tissue has a unique potential for generating genetic variations. In this present study, some of the somaclonal variations are seen by using leaf and stem callus of *Passiflora vitifolia*.

ACKNOWLEDGEMENTS

The authors are thankful to Department of Studies in Botany, University of Mysore for providing the facilities to carrying out this work.

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