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In vitro Cytological Studies of Leaf Callus Cultures of *Orthosiphon aristatus* (Blume) Miq

P. Dorothy[1], M. S. Sudarshana[1], A. R. Nissar[1*] and H. V. Girish[1]

[1]Plant Tissue Culture Laboratory, Department of Studies in Botany, University of Mysore, Manasagangotri, Mysore, Karnataka 570006, India.

Authors' contributions

Author MSS designed the study and the whole study was carried under his guidance and the final manuscript was approved by him. All experimental work, statistical analysis and first draft of the manuscript were managed by authors PD and ARN. Author HVG managed the analyses of the study, literature survey and modified the first manuscript draft. All authors read and approved the final manuscript.

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ABSTRACT

The study was carried out to unravel the *in vitro* cytological behaviour of leaf callus cultures of *Orthosiphon aristatus* (Blume) Miq. The cultures were raised using leaf explants on Murashige and Skoog (MS) medium supplemented with different growth regulators like auxins (2,4-D, IAA, NAA) and cytokinin (BAP) alone and in different concentrations and combinations. Best callusing from the leaf explants was achieved in 2,4-D supplemented MS medium at 5 mg/l concentration. Cytogenetic analysis of the chromosomal variations was carried out for primary cultures as well as 3-month-old callus cultures. The chromosome number did not show variations and the cells studied were observed to be diploid (2n= 28) in nature. Among the numerical observations induced, polyploid cells were the most frequent. The frequency of bridges was observed among the other structural changes. The composition and concentration of the growth regulators was found to affect

**Corresponding author: E-mail: nissarreshi@gmail.com;*

chromosomal instability with multinucleate, multinucleolate and embryogenic masses. These observations indicate that genetic variations may arise during cell cultures.

Keywords: Callus; polyploidy; cytokinesis; multinucleate; enucleated cells.

1. INTRODUCTION

Plants regenerated from the cultured cells are a pre-requisite for the application of somatic cell culture techniques. Although the growth and development *in vitro* is under controlled conditions, the developing cells exhibit variations, abnormalities and deviations from their normal growth phase. Accountable number of research has been undergoing since decades on this significant area of investigation. The stability of *in vitro* culture is a major problem in applying plant cell and tissue culture techniques to basic and applied research. The chromosomal variability can also be a source of somaclonal variation. The source of the explants is considered to be one of the most important factors. The factors like type of culture medium, type of growth regulator and its concentration, age of explant, time duration of callus cultures involved in the stability or variability of *in vitro* cultures have been described in a number of detailed reviews [1,2]. Cytological abnormalities have been found to be responsible for such variations under the promotive influence of chemical as well as physical culture conditions *in vitro* [3-5]. Cytological studies can help to detect the somaclonal variations and genetic stability of a plant when subjected to tissue culture system [6]. Several studies have shown that changes in chromosome number and structure can occur from tissue culture and that chromosomal instability can be induced by media components, culture age, explant tissue, plant genotype, environmental conditions during culture, concentration and type of plant growth regulators in the culture media [7,8]. Knowledge of chromosome structure has played crucial role in the improvement of crop varieties and medicinally important plant species and has far reaching implications [9]. The result of chromosomal studies may also be useful in plant taxonomy and phylogenetic analysis. In the present investigation the chromosomal variations in the callus subculture has been studied. The selected plant *Orthosiphon aristatu*s (Blume) Miq (Lamiaceae) is a rare medicinal plant (shrub) native to Indonesia and is being extensively used in traditional medicine for the treatment of various ailments like antiulcer, anticancer, antifungal and various ailments of kidney [10].

2. MATERIALS AND METHODS

Young and healthy leaf explants of *O. aristatus* were collected from medicinal plant garden, Department of Studies of Botany, University of Mysore. The leaf explants initially washed under running tap water to remove soil and dust particles. The explants were then treated with fungicide bavistin (2%, w/v) for ten minutes followed by washing with sterile double distilled water. Inside the laminar air flow, leaf explants were treated with 0.01% mercuric chloride (w/v) for five minutes followed by washing three times with sterile double distilled water to ensure no traces of mercuric chloride are left. The explants were put on to the sterile blotter discs to drain out the excess water and were cut into the small pieces of size 1 sq.cm. The sterilized and trimmed leaf explants were inoculated onto the MS medium with 3% sucrose and gelled with 0.8% agar as solidifying agent and supplemented with various concentrations of auxins such as 2,4–D, IAA, NAA in combination with cytokinin BAP. The pH of the medium was adjusted to 5.8 before gelling with agar and autoclaved for 20 minutes at 121℃ for 15 minutes. The *in vitro* culture were maintained at 26±2℃ and 60-70% relative humidity, light intensity was 3000 Lux with the photoperiod of 18 hours day light and 6 hours dark.

After 2 weeks the calli were subjected to cytological analysis. The proliferation of same calli was continued and fixed at the end of 8th week.

2.1 Pretreatment

The leaf calli segments were pretreated with 0.02 M 8- Hydroxyquinoline for three and half hours. Then thoroughly washed with water and the calli segments were dropped in Carnoy`s- I fixative 3:1 ratio (Absolute alcohol: Glacial acetic acid) for 24 hours. The calli segments were washed thoroughly in water to remove traces of fixative and stored in 70% alcohol. Staining and squashing: A segment of the callus was dropped 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 (2%) stain for a

minimum of 10 minutes. Stained callus was kept in 0.1% propionic acid before squash preparation. Permanent slides were prepared in butanol: acetic acid (1:1) mixture and mounted in DPX and slides were dried at 60℃ overnight [11].

3. RESULTS

Callus was initiated from the cut ends of the leaves after three weeks of inoculation (Fig .1a). Out of the three auxins used, 2, 4-D was found to be the best for callus induction, inducing profuse callusing at 3 mg/l and 5 mg/ l (Table 1). BAP alone was moderately significant in callus induction when tested up to 5 mg/l (Table 1) Auxins in combination with BAP also were found to induce callus after 3-4 weeks of inoculation. The BAP+IAA (3+1.5 mg/l) combination gave the best callus induction response when compared to other different combinations (Table 2). The calli from all the above combinations were fixed to investigate cytological behaviour.

Fig. 1. Callus proliferation (3 month old)

Cytological studies revealed that the callus cells consisted of enlarged, parenchymatous and meristematic cells with dense cytoplasm and nuclei. Broader range of heterogenecity in the structure of nuclei, asymmetric cell populations with polymorphic nuclei and different ploidy levels were observed. Polyploid cells were encountered frequently. Micronuclei and cytodifferentiating cells were of significant appearance. Cells with different shapes of nuclei were observed. Occasionally enucleated cells were also observed. Presence of two or more nuclei per cell, i.e., multiple nuclei share one common cytoplasm. In a binucleated cell, the nuclei generally were of the same size, but very rarely, they differ in their sizes (Fig. 2a). The callus of one month old was found to contain enucleated cells (Fig. 2b). The normal callus cells were of gigantic size which included enlarged nuclei (Fig. 2c) and the nucleus and chromosome were drawn towards the opposite (Fig. 2d). The embryogenic masses of cells were observed in the 3 month old callus cultures (Fig. 2e). Extensions of papillate projection was the most common feature in a 3 month old callus (Fig. 2f). Initially, a papillate projection appears from the cell wall, and gets separated due to the formation of new cell wall between them, subsequent to the migration of the nucleus along with the cytoplasm. In a 30 day old callus the cells exhibited vascularisation where there is direct transformation of callus cells into tracheids (Fig. 2g). The cells in a callus are parenchymatous in nature and differentiation of these cells occur. In a 2 month old callus cytokinesis was observed where the cell divides to form two daughter cells (Fig. 2h). It usually occurs during the early stages of mitosis. A dividing structure known as the cell plate forms within the centre of the cytoplasm and a new cell wall forms between the two daughter cells (Fig. 2i). A decrease in the frequency of dividing cells and increase in the polyploid cells were seen with increasing age of culture (Fig. 2j). In a 3 month old callus the vessels were seen being deposited with lignin in the form of the transverse rods. This type of thickening is common in xylem vessels and tracheids of protoxylem known as scalariform or ladder- like thickening (Fig. 2k). The thickenings in the cell wall is being deposited in the form of a net or reticulate (Fig. 2l). In such cases the unthickened areas of the cell wall are irregular in shape. The cell wall is more or less uniformly thickened, some small unthickened areas were seen in the form of pits (Fig. 2m).

4. DISCUSSION

Orthosiphon aristatus leaf explants were cultured on to the M.S medium supplemented with different concentrations of auxins (2,4-D, IAA, NAA) alone and in combination with BAP. 2, 4- D was found effective in callus induction where the nature of callus was creamy, nodular and friable which is concurrent with the studies carried out earlier on *Secale cereal* [12]. When BAP was used in combination with different auxins i.e.; (2, 4-D, IAA, NAA), synergistic effect was observed in BAP and IAA combination where profuse callusing was achieved. Synergism between growth regulators have been reported earlier in *Vitex negudo* [13].

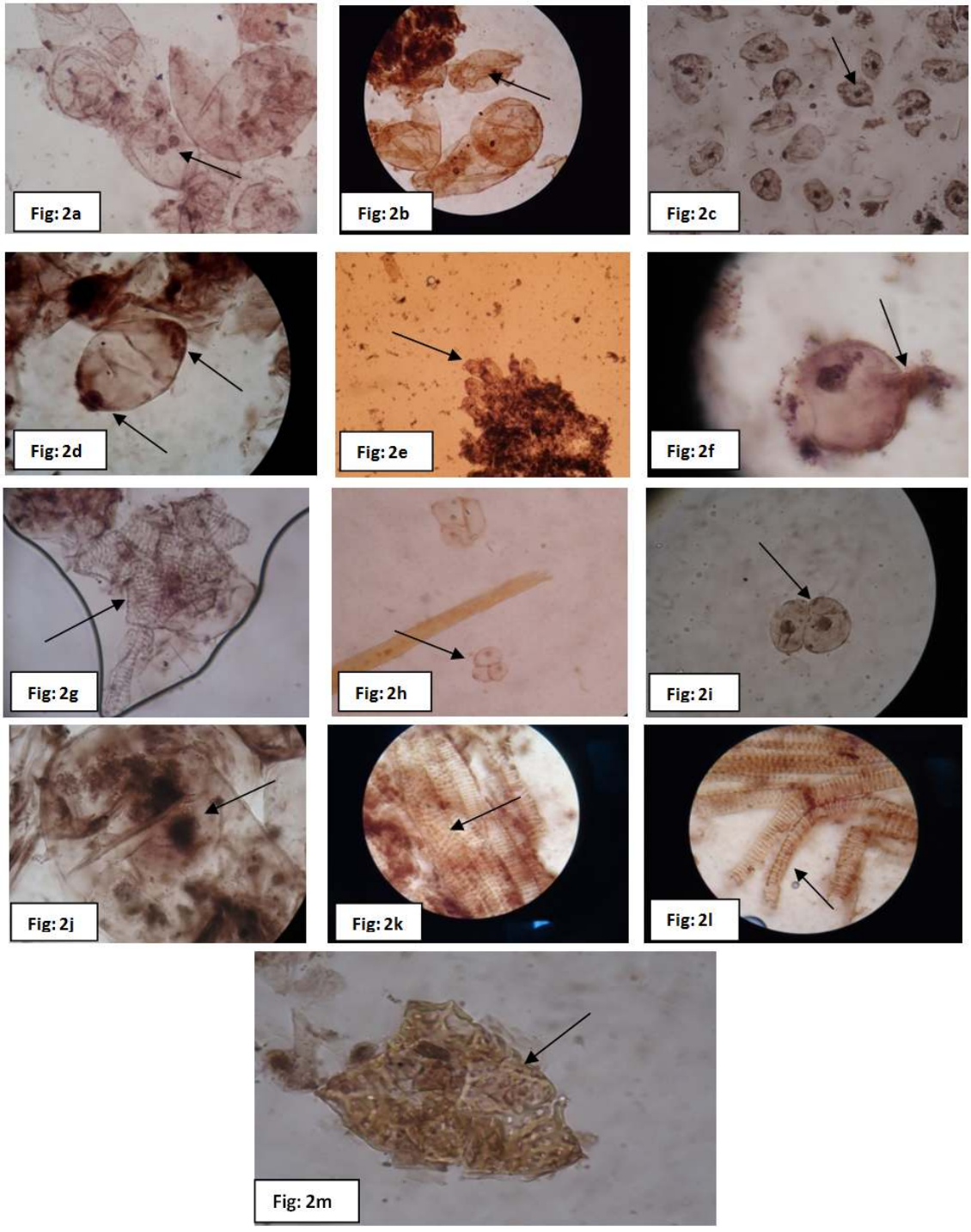

Fig. 2a: Binucleated cell, Fig. 2b: Enucleated cell, Fig. 2c: Normal callus cells with large nucleus Fig. 2d: Single cell with chromosome and nucleus at the polar ends Fig. 2e: Embroyogenic mass, Fig. 2f: Papillate projection, Fig. 2g: Tracheids Fig. 2h: Cytokinesis Fig. 2i: Just divided or separated cell Fig. 2j:, Polyploid cell Fig. 2k: Scalariform type of thickening, Fig. 2l: Reticulate type of thickening, Fig. 2m: Pitted type of thickening

Table 1. Effect of auxins on callusing of leaf explants of *O. aristatus*

Concentration (mg/l)	Callusing % response of leaf explant			
	2,4-D	NAA	IAA	BAP
0.5	50	30	30	00
1.0	60	25	25	20
1.5	65	20	25	25
2.0	70	20	15	30
2.5	65	15	10	40
3.0	80	05	04	35
4.0	80	--	--	30
5.0	82	--	--	25

--No callus, data was collected after 3 weeks with number of explants callused/total number of explants inoculated x100

Table 2. Effect of BAP in combination with NAA and IAA on callusing of leaf explants of *O. aristatus*

Concentration mg/l	% response of leaf explants		
	BAP + 2,4-D	BAP+NAA	BAP+IAA
1+0.5	45	20	15
3+1.5	35	30	70
5+2.5	25	35	25

Data was collected after 3 weeks with number of explants callused/total number of explants x100

The calli showed several types of cells exhibiting cytological variations including non-dividing cells with various, uninucleated cells, binucleated cells, enucleated cells, polyploidy cells, normal dioploid cells, cells showing cytodifferentiation and cytokinesis. Polyploid cells were encountered more frequently and a decrease in the frequency of dividing cells with increase in polyploidy cells were seen with increasing age of cultures which can be seen in the two month old callus of *O. aristatus*. Our results are in accordance with the earlier studies carried on *Trigonella foenumgraecum* L [14].

Embryogenic masses of cells were observed in a 3 month old callus. The cells consisted of conspicuous nuclei and densely cytoplasmic content. They further differentiated into tracheids with pitted, scalariform and reticulate thickenings. The development of thickenings on the walls of callus cells indicates that the differentiation is at the cellular level. As far as the chromosome aberrations are concerned, relatively low level was observed. Among them micronuclei were of significant appearance, while other abnormalities like chromosome bridges in anaphase and chromosome fragments were rarely found. Our studies are in accordance with the studies carried on *Allium commutatum* [15]. Bayliss [16] reported mitotic aberration in tissues incubated *in vitro* during long periods of culture. The chromosome squashes of calli showed most of the cells displaying abnormal mitosis, including changes in chromosome numbers such as polyploidy. Similar mitotic aberrations have been reported in embryonic calli cells of maize, potato, carrot, sunflower [7]

5. CONCLUSION

The present study shows the morphological and genetic influence of different combinations of auxins and cytokinin supplementation of MS media on the callus culture. Since callus culture system offer many advantages as a model system for several biological investigations. The present findings could be used in mass propagation of *O. aristatus* and established callus cultures could be used for the mass production of biologically viable secondary metabolites. The polyploid cells observed could be used as regenerative cells for the production of polyploid plants with enhanced production of biologically active metabolites.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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