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Research Article

CYTOLOGICAL VARIATIONS OF *IN VITRO* STEM CULTURES OF *XANTHOPHYLLUM FLAVASCENS* ROXB. AN ENDANGERED TREE SPECIES OF WESTERN GHATS

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ABSTRACT
Xanthophyllum flavascens is a medicinal small endangered tree to Western Ghats. The cultures were raised using stem explants on MS medium supplemented with different growth regulators like BAP and NAA as well as Kn and 2, 4-D in combinations at 0.5 mg/l each. Cytogenetic analysis of the chromosomal variations was carried out for primary culture as well as 4-month-old callus culture. The chromosome number showed wide range of variation, however, the majority of the cells studied were observed to be diploid (2n= 16) 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 effect chromosomal instability with multinucleate, multinucleolate and cytomixis phenomena. These observations indicated that genetic variations may arise during cell culture.

Keywords: *Stem Callus, Xanthophyllum, Chromosome Bridges, Polyploidy, Cytodifferentiation*

INTRODUCTION
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 involved in the stability or variability of *in vitro* cultures have been described in a number of detailed reviews (Sacristan, 1971; Karp, 1989). 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* (Bayliss, 1980; Larkin and Scowcroft, 1981; Mukhopadhyay *et al.*, 2000). Cytological studies can help to detect somaclonal variations and deduced genetic stability of a plant when subjected to tissue culture system (Raha and Roy, 2003). 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 and even by plant genotype (Lee and Phillips, 1988).
Knowledge of chromosome structure has played crucial role in the improvement of medicinally important plant species and has far reaching implications (Samaddar *et al.*, 2012). The result of chromosomal studies may also be useful in plant taxonomy and phylogenetic analysis. 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 plant regeneration appears to be linked with chromosomal behaviour of the source, callus culture (Tha and Roy, 1982). Therefore there is a need to establish the nature and source of variation in cultured cells. The cultures were maintained upto three subcultures and the cytology was done after every subculture. Factors affecting variation in cultured cells include time in culture, explant source, genotype of the donor plant, environmental conditions during culture, concentration and type of plant growth regulators in the culture media (Sree, 1987). In the present investigation the chromosomal variation in the callus subculture has been studied. The selected plant *Xanthophyllum flavascens* Roxb. (*F. arnottianum* Wt.) (Polygalaceae) is a large shrub or small tree, endemic to the Western Ghats. The bark is used to cure swelling due to viper bite and juice of bark is used for spider toxin.

MATERIALS AND METHODS

Callus cultures of stem explants of *X. flavascens* raised on MS medium supplemented with BAP and NAA were subcultured on the same medium composition. After 2 weeks the calli were subjected to cytological analysis. The proliferation of same calli was continued and fixed at the end of 16th week.

Pretreatment: The leaf calli segments were pretreated with 0.02 Molar 8- Hydroxyquinoline for three and half hours. Thoroughly washed with water and the calli segments were dropped in Carnoy`s- I fixative (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 is dropped in a mordant (2.0% Ferrous ammonium sulphate solution) for 5 minutes. Excess mordant is drained off with 45% propionic acid and stained with propionohematoxylin (2.0%) 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^{0}C overnight.

RESULTS AND DISCUSSION

Results

Table 1: Effect of callus induction from stem explants on MS medium supplemented with different growth hormones

Growth hormones						Callogenesis
Basal medium						--
2,4-D	0.5	1.0	1.5	2.0	2.5	--
NAA	0.5	1.0	1.5	2.0	2.5	--
BAP	0.5	1.0	1.5	2.0	2.5	--
Kn	0.5	1.0	1.5	2.0	2.5	--
	0.5+0.5					++
	0.5+1.0					--
2,4-D+ BAP	0.5+1.5					--
	1.0+2.0					--
	1.5+2.0					--
	0.5+0.5					++
	0.5+1.0					--
	1.0+1.5					--
NAA+ BAP	1.0+2.0					--
	1.5+2.5					--
	0.5+0.5+0.5+0.5					+++
NAA+BAP+2,4-D+ Kn	**1.0+1.0+1.0+1.0**					--

Note: "—" = No response, "++"= Moderate callus, "+++"= Extensive callus

In vitro culture of stem explants on MS medium fortified with BAP and NAA resulted in the formation of callus after 3 weeks of culture. It was found that combinations of BAP and NAA, 2,4-D and BAP, 2,4-D, NAA, BAP and Kn at 0.5mg/l each were most suitable for the production of callus (Figure: a) from stem segments of *X. flavascens* while combination of high concentration of BAP (5.0 mg/l) and NAA (3 mg/l) yielded no response (Table 1). The calli from all the above combinations were fixed to investigate cytological behaviour. Chromosome analysis of callus from primary cultures revealed the presence of normal diploid cells (70%) with 2n=16 chromosomes, and the chromosomes were very small. In 45-day-old callus grown on the same medium, about 50% cells were of 2n composition. Binucleate and trinucleate as well as tetranucleate cells were regular features besides observing normal diploid dividing stages-prophase, metaphase (Figure: b) both equatorial and polar views, anaphase and telophase phases. Long – term maintenance of cultures led to irregular mitosis.

In the 4 month old callus cultures various cytological abnormalities such as endoreduplication in low percent was recorded in *X. flavascens* resulting in polyploid cells. The ploidy number ranged from 4n to 6n. Cells with nuclear projections (Figure: c), chromosomal bridges, asynchrony, cytomixis, multinucleate, multinucleolate as well as flow of nuclear content through the papillate projections were observed alongwith cytodifferentiated cells having various types of thickenings. Cytoplasmic connection between the callus cells can be observed which is evident for the exchange of chromatin materials. The percentage of dividing cells was lower in the old callus than at the beginning of *in vitro* culture. Although the overall rate of cell division was low the evident localized asynchrony was observed (Figure: d) where in a binucleated cell shows 2 different stages – one nucleus at interphase and other at metaphase. The asynchrony occurred especially away from the meristematic centres of the callus. The mitotic index in the individual samplings ranges from 0 to 15%.

Chromosome numbers and morphology were analyzed at regular intervals till the 6^{th} subculture. The population of diploid cells prevailed in the primary callus is 70%. In the 2^{nd} and 3^{rd} subcultures the frequency of diploid cells sharply decreased to 50% and there is an increase in the percentage of bi- and tri- nucleate as well as bi – and tetra nucleolate conditions (Figure: e & f). Polyploid cells (8.0%) gradually increased till the 6^{th} subculture reaching the level of 12.6 % (Figure: g). The frequency of diploid cells, prevailing in the beginning was gradually decreased in the 6^{th} subculture (18.5%).

The cells with more nuclei and nucleoli were frequent in the callus tissues. Generally, the nucleus with one large nucleolus was most frequent. In some callus cells the disturbance of the mitotic spindle was observed. Occasionally anaphase stages had 1-2 bridges (Figure: h) The callus cells (about 6.0%) were found to transform into xylem elements in the subsequent sub- cultures. The medium containing BAP (0.5 mg/l) and NAA (0.5mg/l) was the most efficient to induce xylogenesis (Figure: i). Other abnormalities observed were the occurrence of cytomixis (figure: j) in the cultured cells which led to the formation of aneuploid cells as well as tripolar and multipolar cell formation.

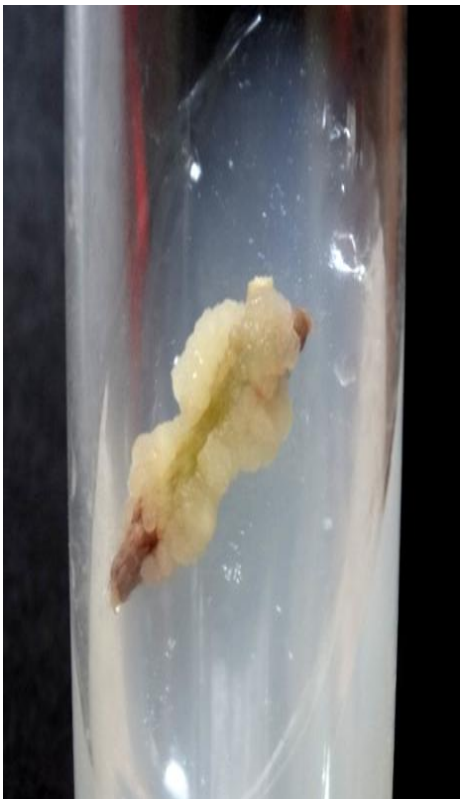

Figure a: Callus proliferation from stem explant

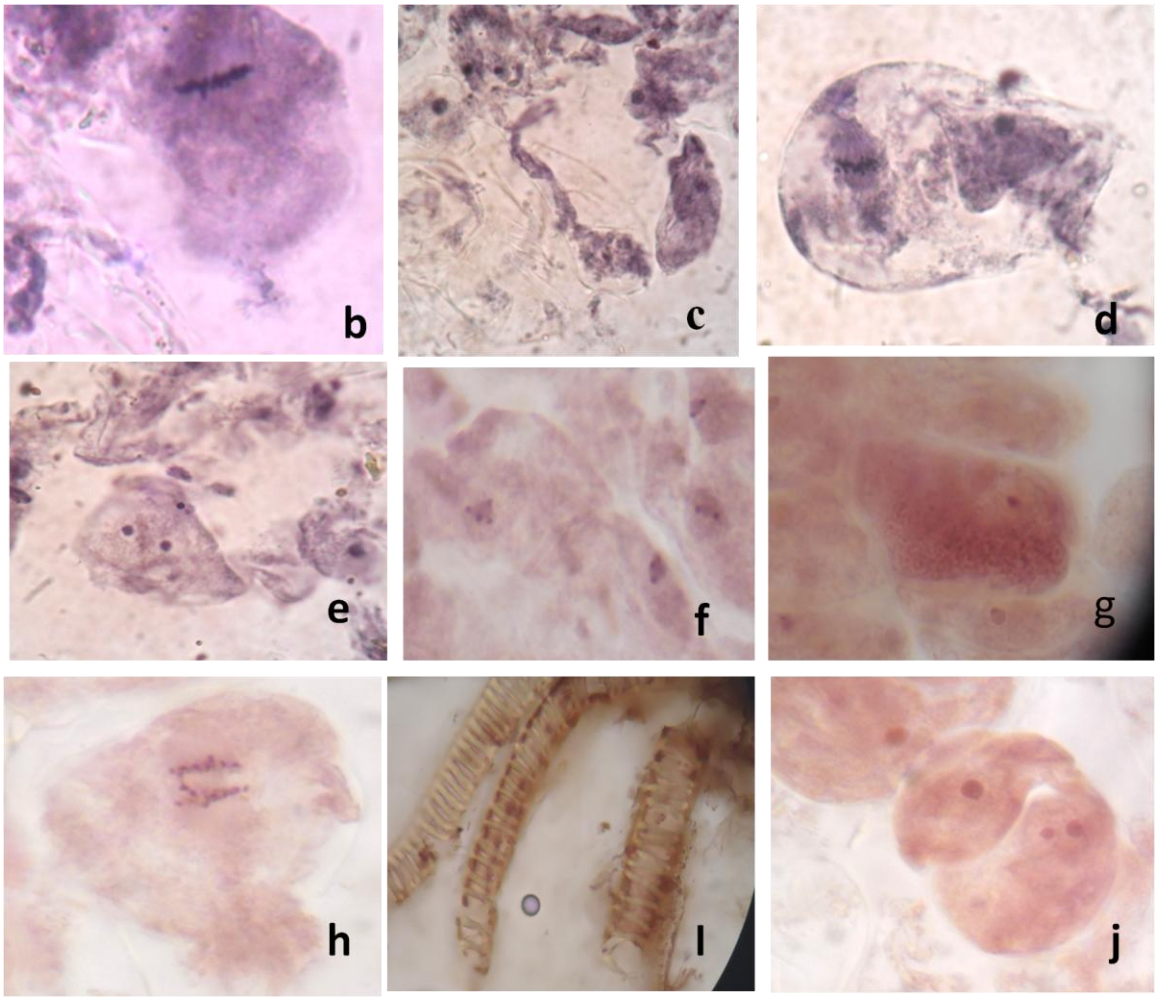

Figure b: Callus cell showing metaphase Figure; c: Callus cell with nuclear projection; Figure d: A binucleate cell with asynchrony showing interphase and metaphase stages; Figure e & f: Bi - and tetra – nucleolate conditions; Figure g: A polyploid callus cell; Figure h: Anaphase with chromosome bridges; Figure i: Xylogenesis of callus cells; Figure j: Callus cell involved in cytomixis phenomenon
Note: From figure b to j observed in 100X

Discussion
Chromosome instability is one of the most common causes of somaclonal variation (Lee and Phillips, 1988). Chromosome variation in tissue culture *in vitro* is well known for many plant species but is still poorly understood. The present study showed that during stem – derived callus of *X. flavascens* a significant decline in the population of diploid cells occurred with concomitant increase in the polyploid cells. This trend was more conspicuous with progressive ageing of the callus cultures. Callus cells with a high ploidy level were observed in tobacco (Shimada, 1971; Fox, 1963), sugarcane (Heinz *et al.*, 1969) and other plants. Chromosome number in callus cells increased as the culture period became longer in a large number of plants (Demoise and Partanen, 1969; Blakey and Steward, 1964).
Occurrence of multi - nucleate cells in the callus cells indicated the possibility of polyploidy. It may also be explained by the pre existing aberrations, which may originate in the tissue used as explant (Karp, 1995). Some species are described as polysomatic plants, because their somatic cells undergo endoreduplication concomitant with the differentiation resulting in mixoploid tissue (D'Amato, 1984). D`Amato (1986) has described several processes: endoreduplication, endomitosis and fusion of nuclei as

the mechanisms of polyploidization in tissues of polysomatic plants cultured *in vitro.* In our investigation in comparison to the primary culture the frequency of polyploid cells was higher in 4 months culture. A steady increase in the frequency of polyploid cells is higher than diploids with the increase in 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.

This can be further substantiated by the fact that a higher percentage of cells in callus tissue exhibited cytological abnormalities like asynchrony, clumping of nucleoli and occurrence of hyperploids and tetraploids may suggest endoreduplication as well. Increasing chromosomal abnormalities with progressive culture age has also been reported earlier (Azam and Biswas, 1989). The variability in nucleoli number and size increased with the successive subcultures probably due to polyploidy observed in some callus cells. The results with respect to cytodifferentiation find support from a number of observations. It has been observed that cytodifferentiation in a diploid condition was encountered frequently (D'Amato, 1977). Inspite of distinct cytodifferentiation in callus culture, organogenesis leading to shoot development has not yet been reported in *X. flavascence.* Along with the presence of variation in chromosome number in callus cultures, a fair amount of structural changes of chromosomes were also observed in all the combinations tried. It was also observed that frequency of anaphase bridges present in the cultures is in agreement with report of Anju and Sarbhoy (1990) in *Pisum*.

In the cultured cells of *X. flavascens* there is the occurrence of cytomixis and it may be a source of production of polyploids and is similar to the work of Yen *et al.,* (1993) in intergeneric hybrids of *Roeyneria cilliaris* and *Psathyrostachys luashanica* and in parallel with the work of Sinha (1988) in *Lindenbergia indica.* Sarvella (1958) is also of opinion that the migration of the genomic complement through cytomixis may be an additional mechanism for the origin of polyploid plants. All the abnormalities may result in reduced potentiality by suppressing regular mitosis.

Cytomixis has been reported in several plant species leading to the formation of aneuploid as well as polyploid cells. Cytomixis leads usually to aneuplody and reduction in fertility of plants. It is therefore considered to be of low evolutionary significance (Sheidai *et al,* 2001; Sheidai *et al,* 2003).

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