Cytological elucidation of somaclonal variation in grasspea (*Lathyrus sativus* L.)

Swapan K. Tripathy*, Ananya Panda and Devraj Lenka

Department of Plant Breeding & Genetics, College of Agriculture, OUAT, Bhubaneswar-751 003, India.

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**ABSTRACT**

A cytological study of root tips, primary calli, subculture calli and regenerants of grass pea (*Lathyrus sativus* L.) cv. Nayagarh local was carried out for elucidation of the origin of tissue culture instability and somaclonal variation. Wide range of euploids, aneuploids and structural variations of chromosomes including stickiness, multipolar spindle, asymmetric chromatid separation, binucleate cells with micronuclei and chromosome bridges and fragments could be the cause for tissue culture instability. Frequency of chromosomal anomalies in calli increased with the age of subculture and the increase was more in B5 medium supplemented with 2, 4-D than NAA. Majority of the abnormalities happens to be sieved off owing to morphogenetic selectivity. While, a few of these were carried on to regenerants as evidenced from mitotic (euploid, aneuploid and structural variation) and meiotic (interchanges and/or loss of chromosome segments following breakage events, lack of pairing resulting univalents, precocious and late separation of bivalents, anaphase laggards, conspicuous anaphase bridges with or without acentric fragments, chromosome rings, tetrad with multinucleoli) abnormalities in regenerants. Such carry-over events have been substantiated as the origin of somaclonal variation in regenerants.

**Key words:** Chromosomal variation, *In vitro* culture, *Lathyrus sativus* L., somaclonal variation.

**INTRODUCTION**

Genetic uniformity and stability of cell and tissue cultures are pre-requisites for their use in somatic cell genetics and genetic transformation. Cytogenetical analysis many often revealed changes occurred in the nuclear material during the process of callus induction and regeneration (Phillips *et al* 1994). Cytological instability of plant cells grown *in vitro* is very common and has been demonstrated by a number of researchers in wide array of plant materials (D’Amato 1975, Sunderland 1977, Bayliss 1980, Orton 1980). Quite often cellular instability is manifested in the form of numerical and structural alteration in chromosomes of cultures as well as plants regenerated from them. However, origin of somaclonal variation still remains far from unequivocal. Some believe it to stem from inherent variation in explants due to point mutation and mixoploidy following endoreduplication or reduplication. Majority of workers, however, opine heritable changes in genome elicited at culture level, due to *in vitro* stress mechanism. The relevant mechanism may best be described as a programmed loss of cellular control. The most commonly observed plant tissue culture-imposed changes could be attributed to chromosome rearrangements (Larkin and Scowcroft 1981), modification in DNA methylation pattern (Phillips *et al* 1994), and transposons mediated mutations (Barret *et al*., 2006; Chen and Henny 2006). Induction of cytological instability was also reported in *Lathyrus sativus* following mutagenesis (Talukdar 2009). Conclusive evidence, whatsoever, is awaited. In this context, *Lathyrus sativus* with fewer number of somatic chromosomes (2n=14) could be an ideal candidate to investigate chromosomal variation in *in vitro* culture and regenerants level as an effect and also as a causal factor itself for inducing somaclonal variation in regenerants.

**MATERIALS AND METHODS**

Different explants from aseptically grown seedlings in basal B5 medium (*Lathyrus sativus* var. Nayagarh local) including root, cotyledon (both whole and sectional), hypocotyl, epicotyls, internode, node, axillary buds, petiole, leaf and shoot tip meristem were tested for their response to callus induction in two selected media, such as, B5 + 2,4-D(2mg/l) + BAP (0.5mg/l) and B5 + NAA(2mg/l) + BAP (0.5mg/l) followed by several subcultures in the same respective medium at an interval of 30days each. Successful regeneration of shoots was obtained in callus precultured in B5 + NAA(2mg/l) + BAP (0.5mg/l) on transfer to B5 + 0.6mg/1IAA + 1mg/lBAP and the regenerated plants were rooted.
on ½ B5 + 1mg/l NAA + 0.02mg/l Kn. While, glossy dark green globular somatic embryos were developed in callus pre-cultured in B5 + 2mg/l 2,4-D + 0.5mg/l BAP on transfer to BM + 0.5mg/l NAA + 2mg/l BAP and whole plantlet with defined shoot and roots were recovered from hormone free MS medium.

Root tips, primary calli and long term embryogenic and organogenetic calli were pretreated with ice cold sterile water for 24 hours followed by saturated solution of parachlorobenzene (PDB) for 4 hours at 4°C and then fixed in glacial acetic acid and 95% ethyl alcohol (1:3) supplemented with a drop of FeCl₃ for overnight at room temperature (27 ± 1°C). Following fixation, materials were transferred to 70% ethanol and stored in refrigerator. For study of chromosomal anomalies in regenerants, root tips and flower buds were fixed in above mentioned fixative. The materials (root tips/anthers/calli particularly hard and nodular calli) were washed in 3-4 changes of distilled water and hydrolyzed in 1 N HCl at 60°C for 10min in a water bath and were squashed in 2% aceto-carmine and observed under stereoscopic microscope for various chromosomal anomalies. The entire research work was carried out at Department of Plant Breeding & Genetics, College of Agriculture, OUAT, Bhubaneswar during the year 2012-13.

RESULTS AND DISCUSSION

Plant tissue cultures have been used extensively to produce genetic variants in crop improvement programme (AdSense, 2010, http://www.articlesbase.com/leadership-articles/somaclonal-variation-3194722.html). Many often phenotypic variations have been correlated with cytological anomalies. A chromosomal aberration may originate either in the explant tissue, or in the sub-clone tissue derived from this explant or even during the process of regeneration. Besides, a variety of factors may contribute to the phenomenon. These include species and genotype, explant source, type of tissue, media components, duration of the culture cycle, age of callus, constitution of callus mass and matrix of culture (organogenic / somatic embryogenic).

Although somatic mutation and residual heterozygosity pre-existing in the cells of donor explants can not be ruled out, somaclonal variation expressed among regenerants and their progeny is a manifestation of genetic changes mostly induced in the callus culture level (De Paepe et al. 1981, Hoffman et al. 1982). Prolonged period of tissue culture increases the frequency of gross chromosomal aberrations (Sanal and Mathur 2004) and regenerants from such long term cultures are expected to have one or more heritable changes (Bairu et al. 2006). In this study regenerants were, therefore, derived from long term callus cultures to obtain wide spectrum of somaclonal variation.

In the present investigation, cytological observations of the callus tissues were performed on the 1st, 2nd and 15th sub-culturing passage. The number of cells at the metaphase and anaphase stage in the cell cycle was shown as the relative activity of the mitotic cell division in each callus tissue cultured on two callus induction media (Table 1). Totipotency of the callus tissues seems to be affected by different media types. The calluses cultured on the medium B5 + 2mg/l 2,4-D + 0.5mg/l BAP showed higher frequency of cell division as well as callus induction frequency (78.2± 0.98%) and rapid callus growth than the medium B5 + NAA (2.0mg/l) + BAP (0.5mg/l). However, the frequency of mitotic cell division though increased in first few sub-cultures, it showed a tendency to decrease with the advanced periods in all cultures.

The root tip cells and primary callus of the source genotype cv. Nayagarh local comprised 14 distinct chromosomes of true-to-the Lythyrus sativus karyotype. In contrast, sub-cultures especially long term cultures from internode explant revealed an array of chromosome anomalies. These anomalies were probably triggered by auxins and other media recipes and subsequent rapid disorganized callus growth (Bayliss 1980). Observation on a series of callus sub-cultures showed heterogeneous mass of dividing cells with a wide range of euploids and aneuploids; and structural variations. After 15 passages (60 weeks), majority of the callus culture cells become virtually mixploidal, higher proportion of which constituted aneuploids (10.8-28.3%) (Table 2). Besides, structural aberrations involved chromosome breakage leading to translocations.

**TABLE 1:** Callus induction frequency(CIF%) and frequency of mitotic cells(%) of calli cultured on two selected callus induction media. Explant : Internode

<table>
<thead>
<tr>
<th>Callus induction Medium</th>
<th>Callus induction frequency(CIF)</th>
<th>Frequency of mitotic cells at diff. stages of subculture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 *</td>
</tr>
<tr>
<td>B5 basal medium(control)</td>
<td>0.0</td>
<td>0.350</td>
</tr>
<tr>
<td>B5 +2mg/l 2,4-D + 0.5mg/l BAP</td>
<td>78.2± 0.98</td>
<td>0.732</td>
</tr>
<tr>
<td>B5 +2mg/l NAA + 0.5mg/l BAP</td>
<td>72.3± 1.12</td>
<td>0.495</td>
</tr>
</tbody>
</table>

*At the time just before transfer to medium for 1st sub-culture.
regenerated from the callus tissues. These facts suggest that higher levels of polyploidy could not be recovered / or chromosome number variations. A few cells of the calli were found to be hyperploid with maximum 40 chromosomes. These might have otherwise hindered caulogenesis and somatic embryo differentiation. Numerical and structural variations in chromosomes of callus cells have also been reported by Tawakley et al. (1992) in Cicer. Thus, presence of an array of chromosome aberrations in long term callus cultures in this study substantiates the observation of De Paepe et al. (1981), Barbier and Dulieu (1983), Lorz and Scowcroft (1983) and Prat (1983) that somaclonal variation in regenerants does originate at culture level.

Morphogenetic calli generally show a balanced chromosome number than the non-morphogenic callus. In the present investigation, cytologically identical aberrations were observed in calli from different passages and plants regenerated at various time span, indicating an early occurrence of the aberration during culture maintenance. High level of cytological anomalies in B5 medium with 2,4-D + BAP (Table 2) is interpreted to be associated with poor caulogenic regeneration potential(Table 3) than calli obtained from B5 medium with NAA + BAP. However, calli pre-cultured in the former was found to be efficient for somatic embryo induction. This could be due to the fact that regeneration via induction of shoot buds and somatic embryos is under separate genetic control and possibly triggered by specific hormones (Tripathy and Cole 2001). In this context, 2,4-D being a strong auxin, contributes towards higher level of chromosomal anomalies by accelerating the rate of polyploidization and other chromosomal variations including structural abnormalities. Polyploidization, especially of higher order, possibly resulted either through DNA endoreduplication (Mohanty et al. 2008), endomitosis, nuclear fusion or spindle failure. Tetraploid plants and much higher levels of polyploidy could not be recovered / regenerated from the callus tissues. These facts suggest that the conditions which are favourable for polyploidization of the calli probably are unfavourable for the morphogenesis of higher levels of polyploids. It may be concluded that in cultured tissues, there is a progressive accumulation of irregularities of chromosomes which increases with the aging process during long term sub-culturing and that these tissues have a reduced potentiality for morphogenesis (Benzion and Phillips (1988).

However, it is still not clear as to why there was coincidence of the two independent phenomena in a common environment e.g., rapid callus growth and the high chromosomal instability in media supplemented with 2,4-D. This might have a certain cause and effect relationship in such a way that the higher callus growth strikingly gave rise to wider variations in the chromosome numbers.

Various other mitotic abnormalities included stickiness, multipolar spindle, asymmetric chromatid separation, binucleate cells with micronuclei, chromosome bridges and fragments (Mujib et al. 2007). Occurrence of chromosome bridges coupled with acentric fragments were more frequent in the long term organogenic cultures and were possibly products of somatic crossing over in inversion heterozygotes. This indicates the possible involvement of a bridge-breakage-fusion cycle phenomenon associated with elicitation of transposable elements under in vitro genome stress (Mc Clintock 1978). These mobile elements by virtue of their transposition along and/or among the chromosomes reinforce the above cycle events and result in a great array of chromosomal aberrations including deletion, duplication, inversion and translocation.

Stickiness was the most abnormality in metaphase preparation of callus culture cells and this reached a maximum up to 36% of mitotic cells. Stickiness may be localized at certain points along the chromosome length or may cover the whole length of chromosomes, causing clotting of chromosomes to a condensed mass. The localized type of stickiness, however, is more likely due to chromosome breakage during sub-cultures.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Stage of subculture</th>
<th>0 *</th>
<th>1st</th>
<th>2nd</th>
<th>15th</th>
</tr>
</thead>
<tbody>
<tr>
<td>B5 basal medium (control)</td>
<td>Eu</td>
<td>Aneu</td>
<td>SV</td>
<td>Eu</td>
<td>Aneu</td>
</tr>
<tr>
<td>B5 +2mg/l 2,4-D + 0.5mg/l BAP</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>B5 +2mg/l NAA + 0.5mg/l BAP</td>
<td>0.2</td>
<td>0.50</td>
<td>0.50</td>
<td>2.50</td>
<td>4.30</td>
</tr>
</tbody>
</table>

*At the time just before transfer to medium for 1st sub-culture.
Eu, Aneu and SV denotes % of cells showing variation in euploidy, aneuploidy and structural rearrangements respectively.
Micronuclei induced in the present investigation could result from the formation of acentric laggards and chromosome fragments which become surrounded by nuclear membranes (El-bayoumi et al. 1979). While, delay or failure of cytokinesis could be associated with occasional occurrence of binucleate cells.

Majority of the chromosomal aberrations induced in callus cultures happens to be sieved off owing to morphogenetic selectivity. For instance, chromosomal variation in euploidy, aneuploidy and structural anomalies in regenerants were observed to be 12.96, 16.6 and 10% respectively (Table 3) as against 18.5, 28.3 and 18.5% in sub-culture calli after 15 passages (Table 2). Some of the variant phenotypes especially those relating to developmental variations, such as, distorted leaf, bifurcated/trifurcated leaf without tendril and slow growth habit were not expressed in R2 generation. Unstability of such variant phenotypes may be ascribed to elimination of chromosomal aberrations that preclude normal morphogenesis. However, chromosomal changes including mutations that carry over into regenerants might be utilized as potentially valuable sources of genetic variation (Larkin and Scowcroft 1981). Cytogenetic changes have been observed in regenerated plants (McCoy et al. 1982 and Ogihara 1981) and these were responsible for some of the observed changes. Other reports included involvement of single gene changes (Edallo et al. 1981, Barbier and Dulieu 1980) and cytoplasmic alterations (Bretell et al. 1980). Random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR) and inter-simple sequence repeat (ISSR) markers have been used to study the genetic fidelity or genetic variability in crops (Sarmento et al. 2005, Gribuado et al. 2009).

Pollen mother cells (PMCs) from the parent variety had normal pairing with seven bivalents in metaphase-I. On an average, 26% of the total regenerants showing structural anomalies carried one or more interchanges and/or loss of chromosome segments following breakage events. Besides, lack of pairing resulting univalents, precocious and late separation of bivalents, anaphase laggards, conspicuous anaphase bridges with or without acentric fragments, chromosome rings, tetrad with multinucleoli were of common occurrence. Chromosomal stickiness, failure of chiasmata to terminalize and delayed separation of chromosome might possibly be the reasons for occurrence of bridges without fragments (Somaroo and Grant 1972). Occurrence of chromosome bridge along with an acentric fragment, however, indicated paracentric inversion. The frequency of fragments and bridges were reported to be highest among other structural changes in callus cultures four pea (*Pisum sativum*) genotypes (Sanal and Mathur 2004). Specific gene effects could result from such chromosomal rearrangements because of gene inactivation, position effects and altered developmental timing (Orton 1983). Despiralization of chromosomes were observed as early as at anaphase II in a few dividing cells (PMCs) of the rosette mutants. This signifies variation in cytological development during meiosis.

Thus, it is clear from the above cytogenetic variation in regenerants that at least a few of the chromosomal anomalies induced in callus cultures, have been perpetuated in the regenerants and such genetic changes along with gene mutations may serve as the basis for the observed phenotypic variation among regenerants.

### TABLE 3: Changes in ploidy level and structural rearrangements in regenerants derived from long term calli (15th passage) pre-cultured in two selected callus induction medium.

<table>
<thead>
<tr>
<th>Callus induction Medium</th>
<th>Regeneration medium</th>
<th>Frequency of regenerated plants with different ploidy level and other chromosomal variations</th>
<th>Total plants with chromo-some number variation</th>
<th>Freq. of plants with structural anomalies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cauilogenic regeneration response (%) in B5 + 0.6mg/l IAA + 1mg/l BAP</td>
<td>14 Ch.(2n)</td>
<td>21Ch.(3n)</td>
<td>48Ch.(4n)</td>
</tr>
<tr>
<td>Basal B5 medium (control)**</td>
<td>0.0</td>
<td>0.0</td>
<td>58</td>
<td>100.00</td>
</tr>
<tr>
<td>B5 + 2mg/l 2.4D + 0.5mg/l BAP</td>
<td>1.5 ± 0.03</td>
<td>4.5 ± 0.25</td>
<td>38</td>
<td>70.30</td>
</tr>
<tr>
<td>B5 + 2mg/l NAA + 0.5mg/l BAP</td>
<td>21.2 ± 1.23</td>
<td>4.0</td>
<td>49</td>
<td>85.96</td>
</tr>
</tbody>
</table>

*-Medium used to raise normal seedling from seeds, **- Figures within the parenthesis denote variation in euploidy.
REFERENCES


