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COMPARATIVE ASSESSMENT OF DIFFERENT PRETREATMENT CHEMICALS AND FIXATIVES DURATION ON MITOTIC SPREAD OF *ALLIUM CEPA* (L.) ROOT CELLS

Animasaun, D.A.*, Oyedeji S., Adedibu P.A. and Abegunde I.F.

Department of Plant Biology, Faculty of Life Sciences, University of Ilorin, Ilorin, Kwara State, Nigeria

Abstract

The study compared two pretreatment liquid substances (ice water and paradichlorobenze), duration of pretreatment and fixatives on the root mitosis of *Allium cepa* to determine the efficiency of the pretreatment and fixatives duration for good and visible mitotic chromosome spreads. *A. cepa* bulbs were grown on sterile water to initiate root formation. Root tips (<0.5 cm) were harvested and pretreated with either Cold Ice Water (CIW) or 0.1% Paradichlorobenzene (PDCB) for 3 and 6 hours. The pretreated root tips were fixed in 1:3 Glacial Acetic Alcohol (GAA) or Propionic Acid Alcohol (PAA) for 24 hours, hydrolyzed with 5-NHCl at room temperature for eight minutes and then stained with aceto-orcein. The slides were viewed under Olympus microscope and photomicrographs of the spread taken. Pretreatment with PDCB for 6 hr and fixed in GAA resulted into better chromosome spread and visibility. Likewise, 6 hr pretreatment with CIW, fixed in PAA produced higher quality chromosome spreads than 3 hr pretreatment. Meanwhile, the use of PAA had greater efficiency than GAA in term of slide qualities as all stages of division were clearly observed. The use of cold ice water as pretreatment for 6 hr coupled with 24 hr fixation in 1:3 Propanoic acid-alcohol produced best spread, had clear and most visible effects on the chromosome of *A. cepa*. The study concluded that even though the two pre-treatment substances inhibited the spindle fibre formation during mitosis and arrested the chromosomes at metaphase, the pretreatment chemicals including the fixatives elicited a differential contraction of the chromosomes. Therefore, pretreatment of root tips with cold ice water coupled with fixation in propanoic acid-alcohol may be developed into protocol for obtaining good and high quality chromosome spread during cytological studies.

Keywords: Chromosome, Fixative, Mitotic spread, Paradichlorobenzene, Pretreatment substance

*Corresponding author e-mail: animasaun.ad@unilorin.edu.ng

Introduction

Cytogenetic analyses in plant has been widely employed in the study of plant evolution, phylogeny, taxonomy and resolution of biosystematics, determination of breeding strategies as well as genes transfer by ploidy manipulation (Chen and Li, 2005). Cytogenetics and chromosome studies requires high quality chromosome spread

preparation, in particular metaphase spread, and is a prerequisite for chromosome banding procedures and in situ hybridization (Mirzaghaderi, 2010).

In most cases, chromosome studies are conducted to determine their number, size and movement during cell division in plant tissues (Kirov *et al.*, 2014). Essentially, chromosome

spread is routinely used to study somatic cell chromosomes, but in practice achieving this sometimes become difficult due to poor spread which could make chromosome counts, determination of size and structural studies a great task (Storey *et al.*, 1968; Chen and Li, 2005). Preparation of good somatic metaphase spreads is hindered by combined difficulties of obtaining large number of dividing cells and of spreading and staining the chromosomes well.

In addition, nature and chromosome morphology are other important factors that affect visibility of chromosomes spread. To achieve a good mitotic chromosome spread, a number of microtubule inhibiting substances have been used as pretreatment chemicals. These include among others colchicine, paradichlorobenzene, ice cold water, 8-hydroxyquinoline and α -bromonaphthalene (Osalou *et al.*, 2013; Ekong *et al.*, 2014).

Though these chemicals have been observed to produce desirable effects pertaining to chromosome condensation and spread, and in addition, their roles in spindle fibres inhibition have also been elucidated by other workers (Burley, 1964). However, there is a dearth of reports on how duration of pretreatment affect mitotic chromosome spread and quality. Analysis of chromosome is one of the major direct methods to count and measure chromosome parameters in plant cells. In order to enable proper chromosome studies, it is necessary for the sample to be in constant mitotic division and *Allium cepa* has been widely used for this purpose due to its ease of storage and growing peculiarities (Smaka-Kincl *et al.*, 1996; Evseeva *et al.*, 2003).

In addition, mitotic and replication indices which are often used as indicators to monitor cell proliferation are easily determined using *Allium cepa* as model.

Although the use of different pretreatment chemicals and fixatives in mitotic preparation has been reported in different studies (Henegariu *et al.*, 2001; Bertao and Aguiar-Perecin, 2002; Anamthawat-Jonsson, 2003), little concern has been given to the detailed protocol of making good quality chromosome spreads by comparing and altering the pretreatment and fixative durations.

Despite several available protocols on plant chromosome preparation, there is no generally applicable method for all plants (Kirov *et al.*, 2014). The difficulty associated with preparation of metaphase plates due to their rigid cell wall and cellular debris usually hampers chromosome character study and to a great extent determines the success of modern cytogenetic techniques such as genomics and fluorescence *in situ* hybridization. The chromosome slide preparation should be well spread, flat and have abundant chromosomes with good morphology, free from cytoplasmic remains and other cellular material (Mirzaghaderi, 2010). For karyotype study, it was observed that variation in pretreatment time affects haploid complement of the chromosome contraction (Mergen and Burley, 1963).

However, the effect of pretreatment chemicals on chromosome characteristics have not been completely elucidated. Knowledge of comparative effects of different pretreatment chemicals could lead to a better understanding of the use of the pretreatments in cytological studies. Therefore, the present study compared the effects of cold ice water and 0.1% paradichlorobenzene as pretreatment chemicals under different time interval as well as the use of glacial acetic alcohol and Propanoic acid-alcohol (PAA) as fixatives. This is to determine the efficiency and optimum duration of pretreatment and fixation on chromosome spread and visibility of the root tip of *Allium cepa*.

Materials and Method

Sample Collection and Germination

Bulbs of *Allium cepa* (onion) used in this study were bought from Ipata market, Ilorin, Kwara State, Nigeria. Wintered and budded onion bulbs were selected, external dried leaves and old roots were removed before the onion bulbs were floated in sterile distilled water in a beaker with the stem disc just touching water to initiate root development. Root tips of about 0.5-1.0 cm long were harvested at 3-5 days after placement on water.

Mitotic Studies

The tender root tips of 0.5 cm were harvested with a pair scissors and collected in labelled specimen tubes. Root tips were treated with different pretreatment substances; aqueous solution of 0.1% Paradichlorobenzene (PDCB) and Cold Ice Water (CIW) for 3 hr and 6 hr. Mitotic slides were prepared as described by Marasek *et al.* (2005) with modifications. At 3 or 6 hr. The pretreated root tips were removed from pretreatment solution, washed twice with distilled water, then fixed in freshly prepared 1:3 Glacial Acetic-Alcohol (GAA) or 1:3 Propanoic acid-Alcohol (PAA) for 24 hrs at room temperature. About 2 mm of the pretreated root tips were hydrolysed in 5-N HCl at room temperature for 8 minutes and squashed in drops of 2% aceto-orcin on a clean glass slides until a turbid suspension was obtained as adapted from Oyewole (1972). The slide was covered with a cover slip which have been previously smeared on one side with a speck of glycerine albumen and passed over

the spirit flame and stored at 4°C until use. Each of the slides was viewed under a light microscope (Olympus).

Photomicrographs of cell division stages were taken for each of the pretreatment substances used and durations with the aid

Micro Image Projection System (MIPS)-Magnus Amscope (MIPS 14F0187; Olympus, USA) fixed on the microscope.

Results

Different stages of mitotic cell division were observed in *Allium cepa* (onion) using 0.1% Paradichlorobenzene (PDCB) or chilled Cold Ice Water (CIW) as pretreatment solution for either 3 or 6 hours. Onion roots pretreated with PDCB for 3 hr and fixed in 1:3 glacial Acetic-Alcohol for 24 hr (PDCB + 3 hr. + GAA) showed mainly cells at prophase with visible chromosomes but tangled and decompressed (Plate 1a & b). The metaphase chromosomes arranged on the equatorial plate, however the chromosome spread overlapped and could not be counted (Plate 1c). Anaphase and telophase were not observed on the slides.

The root tips pretreated with PDCB for 6 hr and fixed in 1:3 glacial Acetic-Alcohol for 24 hr (PDCB + 6 hr + GAA) showed better spread for prophase, metaphase and anaphase (Plate 2). The prophase stage was visible but not distinct, the metaphase spread was visible with countable chromosomes which clearly showed $2n = 16$ chromosome number of onion.

Plate 1: Mitotic spreads of *Allium cepa* pretreated with paradichlorobenzene for 3 hr and fixed in 1:3 glacial acetic-alcohol (PDCB + 3hr + GAA). (a) prophase, P; prometaphase, PrM (b) prophase; P, metaphase; M, and (c) anaphase; A.

Plate 2: Mitotic spreads of *Allium cepa* pretreated with paradichlorobenzene for 6 hr and fixed in 1:3 glacial acetic-alcohol (PDCB + 6 hr + GAA). (a) prophase, P; metaphase, M; (b) metaphase, M; (c) anaphase, A; and (d) anaphase, A

Plate 3: Mitotic spreads of *Allium cepa* pretreated with paradichlorobenzene for 3 hr and fixed in 1:3 propanoic acid-alcohol (PDCB + 3hr + PAA). (a) Prometaphase, PrM; (b) Prometaphase, PrM; (c) prophase, P; metaphase, M; and (d) anaphase, A.

In addition, cells at normal anaphase were observed as sister-chromatids moved towards the spindle poles (Plate 2a & 2d). The PDCB pretreatment of onion root tips for 3 hr followed by fixation in 1:3 Propanoic Acid-Alcohol for 24 hr (PDCB + 3hr + PAA) produced good prophase and prometaphase spreads (Plate 3). The metaphase stage showed clearly chromosomes that are arranged at the equatorial plate. Likewise, distinct anaphase was observed where separated sister-chromatids completely moved towards the spindle poles. The telophase stage was normal, each daughter chromosome arrived at the spindle pole and cytokinesis occurred.

Application of PDCB as pretreatment solution for onion root tips for 6hr, then fixed in 1:3 Propanoic Acid-Alcohol for 24 hr (PDCB + 6hr + PAA) resulted in clear notable

spreads of different division stages (Plate 4). The chromosomes at prophase stage were clear and conspicuous. Meanwhile, clear and distinctive metaphase stage where chromosomes were seen arranged on the equatorial plate was also observed. Anaphase spreads obtained with PDCB + 6hr + PAA, had the sister-chromatids well separated in clumps orientated at the spindle poles. In additions telophase stage with clearly visible binucleated cells were observed (Plate 4). The use of cold ice water as pretreatment for 3 hr followed by fixation in 3:1 ethanol- glacial acetic acid for 24 hr (CIW + 3 hr + GAA) for mitotic studies in onion showed numerous cells at prophase stage (Plate 5).

The metaphase spreads were non-distinctive as the chromosomes clumped at the cells center and cannot be counted, but the observed cells at anaphase were clear with the

chromosomes well separated towards the poles. Also, CIW + 3 hr + GAA treatment produced cells at early telophase where each daughter chromosomes has arrived at the spindle pole in preparation for cytokinesis and separation into distinct daughter cells (Plate 5).

Onion root tips pretreated in ice water for 6hr and fixed in 1:3 Glacial Acetic-Alcohol for 24 hr (CIW + 6hr + GAA) had clear phases of cell division. Although cells at prophase and prometaphase stages were common, good metaphase with chromosome alignment at the cell center were also achieved with the treatment (Plate 6). Many cells were seen at the anaphase, where chromosomes were organised and clearly separated to the spindle poles without numerical alteration or anaphase irregularity.

The cells at final stage of division i.e. telophase were binucleated with nuclear membrane constriction in readiness for cytokinesis (Plate 6). Pretreatment of onion root tips with ice water for 3hr and fixation in 1:3 Propanoic Acid-Alcohol for 24 hr (CIW + 3 hr + PAA) produced good and visible chromosomes spreads.

Several cells were at interphase and prophase stages (Plate 7), but few cells exhibited an irregular metaphase with chromosomes unaligned on the equatorial plate. However at metaphase stage, clear and visible centrally aligned chromosomes were equally seen. The treatment produced cells at the anaphase where separated sister-chromatids moved towards the spindle poles. Likewise, observed cells at telophase showed daughter chromosome arrival at the spindle pole and cytokinesis occurred (Plate 7).

The use of cold ice water for 6hr as pretreatment condition and fixation in 1:3

Propanoic Acid-Alcohol for 24 hr (CIW + 6hr + PAA) resulted into clear and visible prophase and prometaphase (Plate 8). The metaphase spread had visible chromosomes arranged at the equatorial plate, but the spread was too clumpy and not distinct for counting.

However, separated sister-chromatids were seen moving towards the spindle poles at anaphase. The arrival of each daughter chromosome at the spindle pole and cytokinesis which are characteristics of telophase stage were also observed.

Discussion

Chromosome studies have been used severally to understand cell growth and development. Chromosome number and morphology could provide useful information and insight to resolve taxonomic problems (Oyewole, 1972). For years, different pretreatment drugs have been used in the preparation of somatic chromosomes from actively growing root tip (Wagenvoort *et al.*, 1995; Dolezel *et al.*, 1998; Chen and Li, 2005). The results presented in this study showed that pretreatment drugs, time of treatment and choice of fixative affected chromosome spread and visibility.

The use of ice cold water as pretreatment substances couple with use of ethanol-propanoic acid (3:1) produced better result for chromosome spread. It is possible that cold water pretreatment caused stoppage of the DNA synthesis, mitotic activity and simultaneously inhibited microtubule assemblage, thus chromosome become more visible (Olszewska *et al.* 1988). However, pretreating the sample with Cold Ice Water for 3 hr resulted in optimal chromosome spread and clarity. Compared to other methods, combination of ice cold water and propanoic-alcohol as pretreatment and fixative, respectively may cause better disruption of

the karyokinetic spindles which resulted into C-mitotic changes in plant somatic cells (Klein, 1990).

This observation concurred with Chen and Li (2005) who suggested that size and shape of chromosomes are influenced by concentrations of pretreatment chemical and time of treatment. According to Dong *et al.* (2000), pretreatment and fixative chemicals that give clear chromosome spread provide platform for accurate evaluation of the chromosome morphology. In contrast, Dolezel *et al.* (1998) opined that inhibition of mitosis by many pretreatment drugs may cause irregularity of the C-mitosis type, such as multinucleated and restitution nuclei. However, the use of ice water as pretreatment in the present study did not exhibit such irregularities, but for irregular anaphase found in 6 hr treatment time, which of course was a unique phenomenon.

Furthermore, distinct visibility of chromosomes with the ice water pretreatment and fixation in glacial acetic acid was comparable to those of Osalou *et al.* (2013) who observed that ice cold water pretreatment enhances chromosome counts during the mitotic stage in root cells of *Allium cepa*. The authors elucidated that application of α -mono-bromonaphthalene as pretreatment was not encouraging as the chromosomes were difficult to count, unrecognizable and dim. Moreover, the treatment resulted in more diffusion of chromosomes. Ice water as a pretreatment substance and propionic acid as fixative for 3 hr, produced better chromosome spread and visibility than using glacial acetic acid as fixative. This is corroborated by the observation of Pan *et al.* (1992) who obtained better metaphase index using ice water against the use of colchicine as pretreatment drugs. Furthermore, Martens and Reisch (1998) observed that the influence of the time

of sampling on the activity of cell mitosis is very important. Using ice cold water as pretreatment substance for chromosome determination might have positively affected the roots tips during pretreatment.

The pretreatment with 0.1 % paradichlorobenzene for 3 and 6 hr also produced chromosomes that were visible, countable and well spread. Ekong *et al.* (2014) earlier demonstrated that paradichlorobenzene could effectively inhibit the mitotic spindle and arrest the chromosomes at metaphase resulting into good spread. However, the authors submitted that various pretreatment chemical elicits a differential effects on metaphase spread. Lower concentrations of paradichlorobenzene can produce more visible, distinct chromosomes as the mitotic division of cells will be better arrested with increased timing. Higher concentration however may produce toxic effects. Martens and Reisch (1988) observed that the influence of the time of sampling on the activity of cell mitosis is very important.

Conclusion

Different pretreatment drugs produced different results at varying duration of treatment. In the present study, root tip cells of *Allium cepa* pretreated with Cold Ice Water for 3 hr and fixed in Propanoic acid- alcohol for 24 hours produced the better chromosome (mitotic) spread than those pretreated with paradichlorobenzene and fixed in glacial acetic alcohol. The study concludes that pretreatment substances and duration of pretreatment and fixative are important factors that affects the spread and visibility of mitotic chromosomes.

Plate 4: Mitotic spreads of *Allium cepa* pretreated with paradichlorobenzene for 6 hr and fixed in 1:3 propanoic acid-alcohol (PDCB + 6hr + PAA). (a) prophase; p; metaphase; M; telophase, A; (b) anaphase, A; telophase, T.

Plate 5: Mitotic spreads of *Allium cepa* pretreated with ice cold water for 3 hr and fixed in 1:3 glacial acetic-alcohol (CIW + 3hr + GAA). (a) prophase; P; anaphase, A; (b) prophase, P; metaphase, M; (c) anaphase, A; telophase T; and (d) prophase, P; metaphase, M; telophase, T.

Plate 6: Mitotic spreads of *Allium cepa* pretreated with ice cold water for 6 hr and fixed in 1:3 glacial acetic-alcohol (CIW + 6hr + GAA). (a) telophase, T; anaphase, A; (b) prophase, P; metaphase, M; telophase, T; (c) prometaphase, PrM; and (d) prometaphase, P; anaphase, A

Plate 7: Mitotic spreads of *Allium cepa* pretreated with ice cold water for 3 hr and fixed in 1:3 propanoic acid-alcohol (CIW + 3hr + PAA). (a) prophase, P; metaphase, M, anaphase, A; (b) prophase, P; anaphase, A; telophase, T; (c) prophase, P; telophase, T; and (d) interphase, I; metaphase, M; anaphase, A; telophase, T

Plate 8: Mitotic spreads of *Allium cepa* pretreated with ice cold water for 6 hr and fixed in 1:3 propanoic acid-alcohol (CIW + 6hr + PAA). (a) prophase, Pr; metaphase, M; (b) metaphase, M; anaphase, A; (c) prophase, P; metaphase, M; anaphase, A; and (d) prometaphase, PrM; anaphase A

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