Cibtech Journal of Bio-Protocols ISSN: 2319–3840 (Online)
An Online International Journal Available at http://www.cibtech.org/cjbp.htm
2012 Vol. 1 (1) May-August 2012, pp.23-31/Tank and Thaker
Research Article

CHANGES IN DNA AND RNA LEVEL WITH ENDOREDUPLICATION CAN BE DETERMINED USING A-NAPHTHYL RED DYE

Jigna G. Tank and Vrinda S. Thaker
Centre for Advanced Studies in Plant Biotechnology and Genetic Engineering,
Department of Biosciences, Saurashtra University,
Rajkot-360 005, Gujarat (India).
**Author for Correspondence*

ABSTRACT
An easy, rapid and sensitive visible range microplate assay is designed to determine changes in DNA and RNA level with endoreduplication in root tips of *Allium cepa using* α-naphthyl red dye. This process is equally accurate as compared to other intricate methods (morphological differences, stomatal count, chromosomal count in cells, flow cytometry analysis and genetic analysis) used for determination of endoreduplication. It can be used as an alternative for determination of endoreduplication in laboratories not having sophisticated instruments (flow cytometer or advanced microscope). Root tips of *Allium cepa* were treated with colchicine solution to induce endoreduplication in root tip cells. DNA and RNA was isolated and estimated from normal and endoreduplicated root tips using UV absorbance and visible range microplate assay. DNA and RNA concentration obtained from visible range microplate assay was compared with the UV absorbance method in order to prove accuracy and effectiveness of method. Dose dependent increase in the concentration of DNA and RNA was observed in the endoreduplicated root tips than that of normal root tips. Both UV range and visible range microplate methods showed the similar results with same accuracy and sensitivity.

Key Words: *Endoreduplication; Colchicine, Allium Cepa, α-Naphthyl Red, RNA, DNA*

INTRODUCTION
Endoreduplication is a frequent phenomenon in plant organ development. Differentiation of cell for a specialized function in plant tissue is associated with an increase in the DNA content of the cells over the diploid state (Luz *et al.,* 2000). This temporary recurrent duplication of the DNA without mitosis helps in faster growth and development of plant. The organ-specific but reproducible ploidy pattern in plants suggests that endoreduplication cycles are critical for differentiation programs (Grafi 1998). The mechanisms that regulate the switch between cell proliferation and endoreduplication are central to determining cell numbers and cell sizes which finally determine size of organs and plants (Magyar *et al.,* 2005). Sorger *et al*., (1997) showed that the mitotic spindle checkpoint monitors spindle microtubule structure, chromosome alignment on the spindle, and chromosome attachment to kinetochores during mitosis. The spindle checkpoint delays the onset of chromosome segregation during anaphase until any defects in the mitotic spindle are corrected (Gorbsky 1997). Cells which have a defective spindle checkpoint can aberrantly exit from mitosis with a 4N DNA content (Hoyt *et al.,* 1991). These cells may inappropriately continue to the next cell cycle division and enter S phase with 4N DNA content
(Stewart *et al.*, 1999). They undergo one or more extra rounds of chromatid duplication, with a parallel inhibition of mitosis (Seguí-Simarro and Nuez 2008).
For more than 70 years, plant biologists have used colchicine to induce endoreduplication (Lee *et al.,* 2009). It is an alkaloid, originally extracted from plants of the genus *Colchicum*. It disrupts the process of mitosis by halting the process at metaphase (Katzung 1995). It binds with the tubulin dimmers which is responsible for formation of microtubules and inhibits spindle fibers formation (Ramsey and Schemske 1998). Its action is exerted by binding to tubulin dimers, which hampers de novo polymerization of microtubules (MT) and promotes depolymerization of the existing ones, since the tubulin turnover at the (–) end is not compensated at the (+) end of the MT. Therefore, formation and persistence of the mitotic

spindle, phragmoplast or any other MT-based cytoskeletal structure is compromised. This is reflected in most cases in a blockage of mitosis during the assembly of the anaphase spindle (Seguí-Simarro and Nuez 2008). Rizzoni and Palitti (1973) showed that colchicines induce artificial endoreduplication in Chinese hamster cells cultivated *in vitro.* Recently, it has been shown that there are three main types of agent that induce endoreduplication. The first type includes agents that interfere with cytoskeleton assembly (i.e. microtubule inhibitors), e.g., colchicines and colcemid (Cortes *et al.,* 2004). The second type consists of topoisomerase II inhibitors, which includes both enzyme poisons (i.e., etoposide, amsacrine, and adriamycin) and catalytic inhibitors (i.e., merbarone, aclarubicin, and ICRF- 193) (Joubes and Chevalier 2000; Inze and De Veylder 2006). The third type comprises physical and chemical agents that damage DNA, such as X-rays. Some of these microtubule-interfering agents, such as nocodazole and paclitaxel, induce significant endoreduplication because sister chromatid segregation is interrupted (Andreassen *et al.*, 1996; Jordan *et al.*, 2003). It has been shown that the level of cyclin B-like proteins increases in colchicine-arrested metaphase cells (Chaudhuri and Ghosh 1997). On the other hand, endoreduplication and polyploidy occur in cells expressing undegradable cyclin B (Weingartner *et al.,* 2004). Sadhan et al (1997) also showed that the level of cyclin B proteins remained high in colchicine arrested metaphase cells of *Allium cepa*. Hence, in the present studies roots of *Allium cepa* were exogenously treated with colchicine to induce endoreduplication in root tip cells. Endoreduplication increases the number of chromatids of a chromosome without a change in the number of chromosomes. If more than one duplication round takes place, polytene chromosomes are formed. Diplochromosomes or polytenic chromosomes are generally originated without the usual rounds of chromatin condensation and decondensation (Sugimoto-Shirasu and Roberts 2003), which makes it very difficult to gain insights about their structure. Although, it is determined by several laboratory techniques such as morphological differences, stomatal count, chromosomal count in cells, flow cytometry analysis, and genetic analysis (Cramer 1999; Besnard *et al.*, 2007; Bourdon *et al.*, 2011). But all this techniques are very lengthy and require sophisticated instruments. Thus, an attempt has been made to develop an easy and rapid method for determination of endoreduplication in root tip cells of Allium cepa using simple visible range microplate reader.

MATERIALS AND METHODS

Induction of Endoreduplication

Onion bulbs of uniform size were selected; dry leaves were removed and washed thoroughly with water. Bulbs were then kept in a tray filled with distilled water for 3 days at 25 oC temperature for development of young roots. They were then placed in coupling jars filled with colchicine solution of different concentrations viz, 0.01%, 0.03%, 0.05% and 0.1%. Roots were incubated up to 120h in colchicine solution to develop endoreplication at utmost level. Roots grown in distilled water were considered as control.

Cytological Analysis

The cells undergoing mitosis and endoreduplication were stained with fuschin stain and observed under Carl Zeiss light microscope. The size of cells and nucleus was measured by using imaging power of Axiovision 4 software.

Molecular Analysis

Tips of endoreduplicated and control roots were cut. DNA was isolated from them, following the Alkali lysis method suggested by Remi *et al.*, in 2000. Root tips (1 gm) were taken and crushed in chilled condition by adding 7 ml of extraction buffer (200mM Tris-Hcl, 50mM EDTA, 1% SDS, pH- 8.0). Crushed material was incubated at 37 ^{o}C for 30 minutes. The tubes were kept in horizontal manner and shaked vigorously at an interval of 5 minutes. Shaking reduces aggregation of material and enhances DNA release. After incubation crushed tips were centrifuged at 5000 g for 15 minutes at 4 ^{o}C. Pellet containing cellular debris and high molecular weight polysaccharides was separated. Supernatant was collected and RNase (25 µl) was added and incubated for 30 minutes at 37 ^{o}C. This step allows RNA

degradation. Afterwards sample was transferred on ice and incubated for 30 minutes with occasional shaking by gently inverting the tubes 3 times. This incubation enhances the precipitation of large amount of polysaccharides and proteins as salt complexes. Precipitated contaminants were removed by centrifugation at 5000 g for 15 min at 4 ^{o}C. Further purification of samples was achieved by mixing Phenol: Chloroform: Isoamyl Alcohol (24: 25: 1) with equal volume of samples. Repetition of this step 3 times enhances the DNA purity by removal of contaminants through phase separation. Chloroform causes surface denaturation of proteins but it has no effect on solubility of DNA. Whereas isoamyl alcohol reduces foaming, aids separation and maintains the stability of two separated layers. Subsequent centrifugations at 5000 g for 15minutes at 4 ^{o}C helped to remove the denatured proteins from soluble DNA. Chilled isopropyl alcohol (-20 ^{o}C) was mixed with sample solution (1:1) to achieve DNA precipitation. This was again centrifuged under same conditions to pellet the DNA. Supernatant was discarded and DNA pellet was washed thrice with 1 ml of -20 ^{o}C cold 70 % ethanol with repeated centrifugation after each wash. Ethanol lowers the effective water concentration, causing large biomolecules to interpenetrate and aggregate. Pellets were then allowed to air dry in order to evaporate the ethanol. They were resuspended in 300 µl of TE buffer for prolong storage and subsequent experiments. Purity of DNA was determined by measuring its optical density in spectrophotometer at A260 nm/ A280 nm ratio and concentration of DNA samples were determined by measuring its optical density at A260 nm. Quality of DNA samples was checked by loading them on 1% agarose gel and observing it on UV illuminator.

Isolation of RNA from Root Tips of Allium Cepa

RNA was isolated from normal and endoreduplicated root tips of *Allium cepa*. 1 gm root tips were homogenized in Tri-reagent (0.5ml) using mortar and pestle. Homogenized samples were allowed to stand for 20 min. at room temperature to ensure complete dissociation of nucleoprotein complexes. Chloroform (0.2 ml) was added per ml of Tri-reagent used to all samples and shaked vigorously for 15 seconds. They were allowed to stand for 15 minutes at room temperature. Centrifugation of the resulting mixture was done at 12,000 Xg for 15 minutes at 2-8 °C. Centrifugation separated the mixture into 3 phases: a red organic phase (containing protein), an interphase (containing DNA), and a colorless upper aqueous phase (containing RNA). The aqueous phase was transferred to a fresh tube and isopropanol (0.5 ml) was added it. Mixture was allowed to stand for 10 minutes at room temperature. The precipitated RNA was pelleted by centrifugation at 12,000 Xg for 10 minutes at 2-8 ^{o}C. RNA pellet was washed by adding 100% ethanol, vortexing the sample and centrifuging it at 7,500 Xg for 5 minutes at 2–8 °C. Further, RNA pellet was air dried for 5–10 minutes. Appropriate volume of TE buffer was added to the RNA pellet to facilitate dissolution and mixed properly by repeated pipetting with a micropipette at 55–60 ^{o}C for 10–15 minutes to facilitate dissolution. Purity and concentration of
RNA was determined by measuring optical density at 260 nm and 280 nm. RNA concentration wasdetermined using following formulae: Concentration of RNA in normal root tip sample = 40µg x A260 x dilution factor

Denaturing Agarose Gel Electrophoresis for RNA

Quality of total RNA was determined by running it on 3% denaturing agarose gel. 0.3 gm of agarose was dissolved in 10ml of 1X TAE buffer with the help of water bath. 3µl of ethidium bromine (10 mg/ml) was added to it and mixed properly. Gel was poured in gel caster and was allowed to solidify. Solidified gel was transferred in electrophoresis unit filled with running buffer. RNA samples were mixed with gel loading buffer (0.25% Bromophenol Blue, 40% sucrose, 2% glycerol, 20% urea). Mixture was loaded carefully into the wells placed on cathode by using micropipette. Gel tank was covered with lid and then electrophoresis run was carried out at constant 50v DC electric pressure. After completion of run, gel was examined on UV transilluminator and photographed for documentation. Figure 2 shows the bands of RNA samples on 3% agarose gel.

Spectra Analysis to Differentiate DNA and RNA Samples in Visible Range Wavelength:

DNA and RNA samples isolated from normal root tips of *Allium cepa* were stained with naphthyl red dye at pH-5 and pH-7. Spectral analyses of both samples were taken at UV-visible range wavelength (360-700nm). Graph of Optical density vs wavelength was plotted and absorbance maximum of RNA and DNA samples were determined.

Colorimetric Microplate Assay:

DNA samples (100 µl) were stained to magenta color by adding 5 µl of 1mg/ml α-naphthyl red dye. Optical density of the samples was measured at A545 nm at pH-7 using the µQuant microplate reader. Concentration of DNA from normal and endoreduplicated root tips was determined by preparing calibration curve using calf thymus DNA (range 20-100 µg/ml) as a standard (Figure 1).

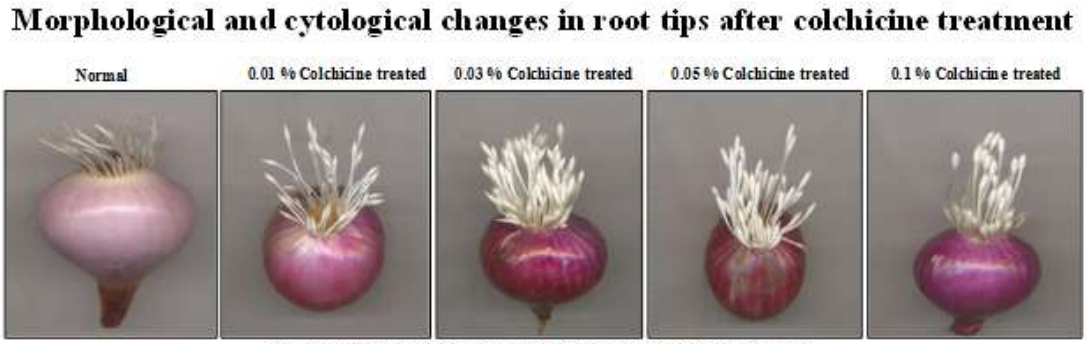

Figure: 1 C-tumor formation in colchicine treated roots of *Allium cepa*

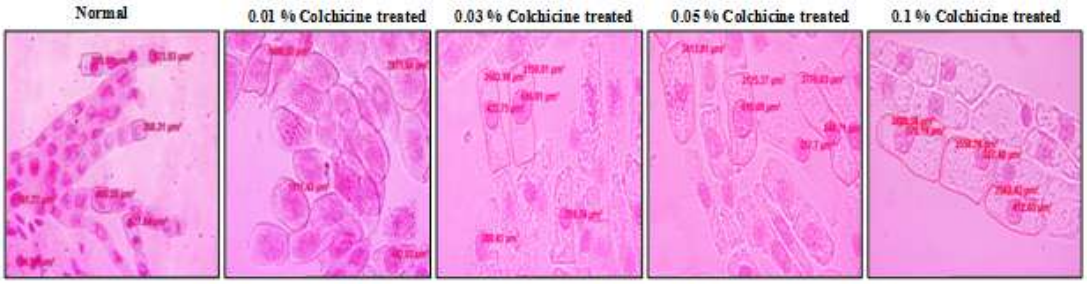

Figure: 2 Cell and nucleus size of normal and endoreduplicated root tip cells of *Allium cepa*

Statistical Analysis

Data was further statistically evaluated by Analysis of Variance (Single factor ANOVA) and Correlation between cytological assay, molecular assay and Colorimetric microplate assay. Analysis was carried out in Microsoft Excel 2003.

RESULTS

Morphology and Cytology of Endoreduplicated Roots

Exogenous colchicine treatment inhibited growth of roots and resulted in formation of C-tumor at the tip of *Allium cepa* root. C-tumor formation was observed at the tip of root after 24 h of colchicines treatment. Figure 1 shows c-tumor formation in root tips of Allium cepa after treatment with different concentrations of colchicine. Dose dependent response of colchicine in induction of endoreplication in root tip cells was observed. There was gradual increase in thickness of c-tumor with increase in concentration of colchicine from 0.01% to 0.1%. Colchicine influenced the cell division status in root tip cells by promoting DNA replication and inhibiting cytokinesis. There was remarkable increase in cell and nucleus size of root tip cells after colchicine treatment as compared to normal root tip cells. Figure 2 shows increase in cell and nucleus size of root tip cells after colchicine treatment.

Estimation of DNA and RNA Level Using UV Asorbance Method

DNA and RNA were isolated from the normal and endoreduplicated root tips of *Allium cepa*. Concentration of DNA and RNA was determined by measuring optical density at 260 nm. Optical density

at 260 nm is a common wavelength for measurement of nucleic acids (DNA and RNA). There was a clear increase in DNA and RNA concentration with increase in colchicine concentration. In normal root tip cells, DNA level was 27.22 µg/ml and RNA level was 8.40 µg/ml, whereas in endoreduplicated root tip cells it showed dose dependent response. In lower colchicine concentration (0.01%) treated root tips DNA level remained 67.36 µg/ml and RNA level remained 25.06 µg/ml which increased in a parallel manner to 105.91µg/ml of DNA and 78.5 µg/ml of RNA with increase in colchicine concentration (0.1%).
Since, Agarose gel electrophoresis separates the DNA bands on the basis of the molecular weight. Normal and endoreduplicated DNA and RNA samples were loaded on 1% and 3% agarose gel respectively. There was remarkable increase in intensity of DNA and RNA bands in colchicine treated samples than that of untreated normal root samples (Figure 6 and 7).

Agarose gel electrophoresis of DNA and RNA samples

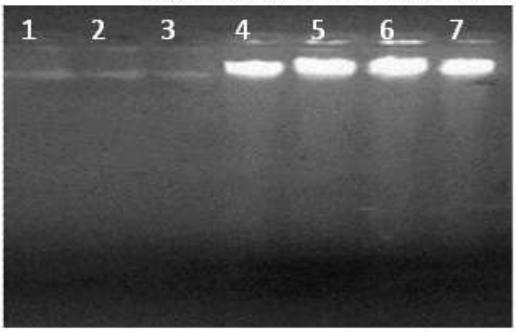

Figure: 6 Polyploid and normal DNA samples loaded on 1% agarose Gel

Lane 1, 2 & 3	DNA band from normal root tips
Lane 4,5, 6 & 7	DNA band from colchicine treated root tips

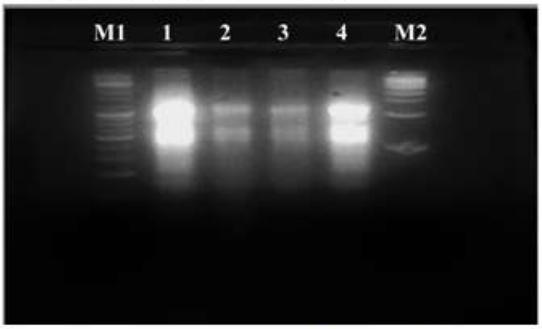

Figure: 7 Polyploid and Normal RNA samples loaded on 3% agarose Gel

Lane M1 & M2	RiboRuler
Lane 1 & 4	Total RNA isolated from colchicine treated root tips
Lane 2 &3	Total RNA isolated from normal root tips

Estimation of DNA Level Using Visible Range Microplate Reader
Concentration of DNA isolated from normal and endoreduplicated root tips was also determined by using visible range microplate reader. Microplate assay showed significant variation in the DNA level of endoreduplicated and normal root tip cells. Endoreduplicated roots showed double amount of DNA and RNA level than that of normal roots. DNA and RNA level in normal root tips was 27.94 µg/ml and 8.55 µg/ml respectively, which increased in endoreduplicated root tips to 104.88 µg/ml and 78.25 µg/ml.
Spectral Analysis of DNA and RNA Samples Isolated from Normal Root Tips of Allium Cepa
DNA and RNA samples stained with naphthyl red dye at pH-5 showed remarkable change in chromatism. RNA sample had pink color and DNA sample had magenta color (Figure 3).

Samples of DNA and RNA stained with α-naphthyl red dye

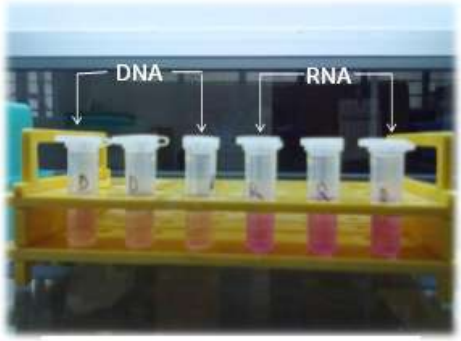

Figure: 3 Change in color of DNA and RNA samples

Spectra of DNA samples at pH-5 showed absorbance maximum of 490 nm whereas, spectra of RNA samples at pH-5 showed absorbance maximum of 530nm (Figure 4). When pH of this samples were changed to 7 then, absorbance maximum of DNA sample shifted to 550 nm and for RNA sample it shifted to 460 nm (Figure 5).

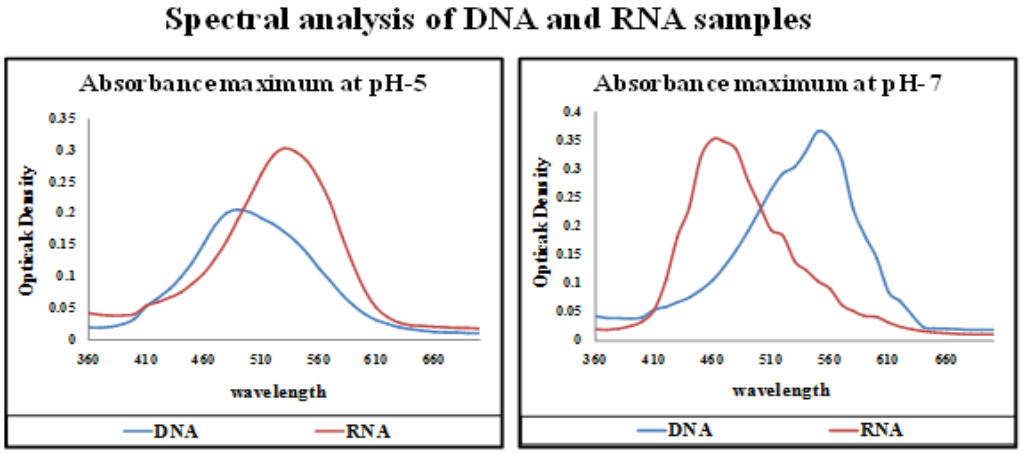

Figure: 4 Spectra analysis of DNA and RNA samples at pH-5 in UV-Visible range wavelength

Figure: 5 Spectra analysis of DNA and RNA samples at pH-7 in UV-Visible range wavelength

DISCUSSION

DNA and RNA was isolated from normal and endoreduplicated root tips of *Allium cepa* to determine the change in DNA and RNA level due to endoreduplication process induced within root tip cells. Here, two methods were employed to estimate DNA and RNA level from normal and endoreduplicated root tips. First, UV absorbance method was used to measure DNA and RNA level as it is the universal method used to measure nucleic acids. Second, microplate visible range assay was used to determine DNA level from normal and endoreduplicated root tips. Naphthyl red dye was used to stain DNA, as its dye conjugated to DNA, shows distinct chromism by hybridization with its complementary DNA. The change in chromism of naphthyl red dye depends on the pH of the sample solution. Naphthyl red dye changes its color from orange to magenta by the protonation of the azo group at low pH (Buvari and Barcza 1989). Deprotonated naphthyl red in the single-strand and duplex gave λ max at 460 and 510 nm, respectively. At around pH 7, an increase in the p*K*a of naphthyl Red by hybridization enhanced chromism. Naphthyl red is partially protonated in the duplex and is deprotonated in the single strand. Asanuma *et al.*, in 2003 showed that the negatively charged environment provided by the deprotonated phosphodiester linkages in the duplex caused this large bathochromic shift and the change of p*K*a by hybridization. These anions on phosphodiesters would decrease the electron density on the dimethylamino group and raise the density on the azo group, which contributed to both the large bathochromic shift and the increase in p*K*a (Letsinger and Schott 1981; Asanuma *et al.*, 2001). Double stranded DNA involving the naphthyl red dye exhibits magenta color and has λ max at 545 nm at pH 7.0. When this double stranded DNA are denatured to single-stranded DNA by heating at 95 ^{o}C its absorption maximum shifts towards 466 nm at pH 7.0 and the naphthyl red dye exhibits an orange color. Hence, the color of the solution changes from magenta to orange accordingly (Asanuma *et al.*, 2003). DNA samples were stained with naphthyl red dye because it has capacity to form complex with DNA. It intercalates between the base-pairs of DNA (Fujii *et al.*, 2007). As, naphtyl red dye binds to the oligonucleotides and gives sharp single peak at absorbance maxima 545 nm at pH 7.0, its optical density is directly proportional to the concentration of DNA present in the sample (Asanuma *et al.*, 2003). The absorbance maxima 545 nm at pH 7.0 was used to determine DNA level in normal and endoreduplicated roots. Hence, DNA samples of normal roots and endoreduplicated roots when stained with α-naphthyl red dye showed magenta color as they were double stranded and its optical density was measured at 545 nm at pH 7.0.

However, there arises one question that whether RNA contamination interferes during measurement of DNA level by colorimetric microplate assays and gives false analysis. Since, the chemical structure of RNA is very similar to that of DNA, with two differences: (a) RNA contains the sugar *ribose*, while DNA contains the slightly different sugar *deoxyribose* (a type of ribose that lacks one oxygen atom), and (b) RNA has the nucleobase uracil while DNA contains thymine. Uracil and thymine have similar base-pairing properties. To solve this problem, DNA and RNA samples were stained with naphthyl red dye at pH 5.0 and pH 7.0. UV-Visible spectra of stained DNA and RNA samples were taken. When UV-Visible spectra of stained DNA and RNA samples were measured at pH 5.0, its absorbance maximum was 490 nm and 530 nm respectively. This absorbance maximum shifted to 550 nm at pH 7.0 in DNA samples and 460nm at pH 7.0 in RNA samples. Unlike DNA, most RNA molecules are singlestranded. Single-stranded RNA molecules adopt very complex three-dimensional structures, since they are not restricted to the repetitive double-helical form of double-stranded DNA. Deoxyribose sugar in DNA is less reactive because of C-H bonds on the second carbon (C2). Hence, DNA is stable in alkaline conditions. On the other hand, RNA, ribose sugar is more reactive because of the presence of hydroxyl group on C2. RNA is not stable in alkaline conditions because bases can easily protonate the hydrogen from the -OH on C2. After protonation, the negatively charged oxygen may attack the Phosphate at the PO4, kicking off the Oxygen connected to the 5'C of next nucleotide over, resulting in hydrogenation (Lesnik *et al.*, 1995). It is also assumed that the change in chromitism of naphthyl red dye was also due to shift in pKa value of RNA samples at pH-5 and pH-7. Raising the pKa of adenine and cytosine above eight, while at a physiological pH of 7, would make them positively charged and carrying an additional proton. Lowering the pKa of guanine or uracil below six, again at physiological pH of 7, would leave them deprotonated and negatively charged (Saenger 1984; Moody et al 2005).

REFERENCES

Andreassen PR, Martineau SN and Margolis RL (1996). Chemical Induction of Mitotic Checkpoint over Ride in Mammalian Cells Results in Aneuploidy Following a Transient Tetraploid State. *Mut. Research* **372**(2) 181-194.

Asanuma H, Kashida H, Lianga X and Komiyama M (2003). DNA Naphthyl Red Conjugate as a Visualizing Probe of DNA Hybridization. *Chemistry Communication* **13** 1536–1537.

Asanuma H, Takarada T, Yoshida T, Liang X and Komiyama M (2001). Enantioselective Incorporation of Azobenzenes Into Oligodeoxyribonucleotide for the Effective Photo Regulation of Duplex Formation. *Angew. Chemistry International Edition* **40**(14) 2671-2673.

Awoleye F, Van DM, Dolezel J and Novak FJ (1994). Nuclear DNA Content and in Vitro Induced Somatic Polyploidization for Casssava (*Manihot Esculenta, Crantz*) Breeding. *Euphytica* **76**(3) 195-202.

Besnard G, Garcia Verdugo C, Rubio De Casas R, Treier UA, Galland N and Vargas P (2008). Polyploidy in the Olive Complex (Olea europaea): Evidence from Flow Cytometry and Nuclear Microsatellite Analyses. *Anatomy Botany* **101**(1) 25–30.

Bourdon M, Coriton O, Pirrello J, Cheniclet C, Brown SC, Poujol C, Chevalier C, Renaudin J and Frangne N (2011). In Planta Quantification of Endoreduplication Using Fluorescent in Situ Hybridization (FISH). *Journal of Plant Science* **66**(6) 1089-1099.

Buvari A and Barcza L (1989). Colour Change and Tautomerism of Some azo-Indicators on Complex Formation with Cyclodextrins. *J. Inclusion Phenom Mol. Recognit. Chemistry* **7**(3) 313–319.

Chaudhuri SK and Ghosh S (1997). Monoclonal Antibody Raised Against Human Mitotic Cyclin B1 Identifies Cyclin B like Mitotic Proteins in Synchronized Onion (*Allium Cepa*) Root meristem. *Cell Biology International* **21**(3) 159-166.

Cortes F, Mateos S, Pastor N and Dominguez I (2004). Towards a Comprehensive Model for Induced Endoreduplication. *Life Science* **76**(2) 21-35.

Cramer CS (1999). Laboratory Techniques for Determining Polyploidy in Plants. *Hort Technology* **9**(4) 594-596.

Fujii T, Kashida H and Asanuma H (2007). Preparation of "Comb Type" Hetero Aggregates by DNA-Dye Conjugation. *Nucleic Acids Symp. Series* **51** 277-278.
Gorbsky GJ (1997). Cell Cycle Checkpoints: Arresting Progress in Mitosis. *BioEssays* 19 193-197.
Grafi G (1998). Cell Cycle Regulation of DNA Replication: the Endoreduplication Prospective. *Exp. Cell Research* **244**(2) 372-378.
Hancock JF (1997). The Colchicine Story. *Horticulture Science* **32**(6) 1011-1012.
Hoyt MA, Totis L and Roberts BT (1991). *S. Cerevisiae* Genes Required for Cell Cycle Arrest in Response to Loss of Microtubule Function. *Cell* **66**(3) 507– 517.
Inze D and Lieven DV (2006). Cell Cycle Regulation in Plant Development1. *Ann. Rev. of Gen.* **40** 77-105.
Jordan MA, Toso RJ, Thrower D and Wilson L (1993). Mechanism of Mitotic Block and Inhibition of Cell Proliferation by Taxol at Low Concentrations. *Proc. National Academy Science* **90**(20) 9552-9556.
Joubes J, Chevalier C, Dudits D, Heberle-Bors E, Inze D, Umeda M and Renaudi JP (2000). CDK-Related Protein Kinases in Plants. *Plant Mol. Biology* **43**(5-6) 607-620.
Katzung BG (1995). Basic and Clinical Pharmacology. *Apleton and Lange: Norwalk*
Lagunes-Espinoza LC, Huyghe C, Bousseau D, Barre P and Papineau J (2000). Endoreduplication Occurs During Pod Wall Development in Temperate Grain Legumes. *Ann. Botany* **86** 185-190.
Lee HO, Davidson JM and Duronio RJ (2009). Endoreplication: *Polyploidy with Purpose. Genes and dev.* **23**(21) 2461-2477.
Lesnik EA and Freier SM (1995). Relative Thermodynamic Stability of DNA, RNA, and DNA: RNA Hybrid Duplexes: *Relationship with Base Composition and Structure. Biochemistry* **34**(34) 10807-10815.
Letsinger RL and Schott ME (1981). Selectivity in Binding a Phenanthridinium-Dinucleotide Derivative to Homopolynucleotides. *Journal of Am chemistry Sociology* **103**(24) 7394-7396.
Magyar Z, De Veylder L, Atanassova A, Bako L, Inze D and Bogrea L (2005). The Role of the *Arabidopsis* E2FB Transcription Factor in Regulating Auxin-Dependent Cell Division. *The Plant Cell* **17**(9) 2527–2541.
McGowan J and Bishop CJ (1953). A Cytological Study of Colchicine-Induced Nodal "Tumors" in Tradescantia. *Cancer Research* **13**(9) 646-650.
Moody EM, Lecomte JT and Bevilacqua PC (2005). Linkage Between Proton Binding and Folding in RNA: A Thermodynamic Framework and its Experimental Application for Investigating P*ka* Shifting. *RNA* **11**(2) 157-172.
Ramsey J and Schemske DW (1998). Pathways, Mechanisms and Rates of Polyploidy Formation in Flowering Plants. *Annual Rev. Ecology Evol. Syst.* **29** 467-501.
Remi W, Paulo AP and Christine AM (2000). DNA Isolation Protocol for Red Seaweed (*Rhodophyta*). *Plant Mol. Biology Rep.* **18**(3) 275–281.
Rizzoni M and Palitti F (1973). Regulatory Mechanism of Cell Division: I. *Colchicine-induced endoreduplication. Exp. Cell Research* **77**(1-2) 450-458.
Sadhan KC and Sibdas G (1997). Monoclonal Antibody Raised Against Human Mitotic Cyclin b1, Identifies Cyclin B-Like Mitotic Proteins in Synchronized Onion (*Allium Cepa* l.) *Root Meristem Cell Biology Int.* **21**(3) 159–166.
Saenger W (1984). Principles of Nucleic Acid Structure. *Springer advanced texts in chemistry*, Springer-Verlag, New York
Seguí-Simarro JM and Nuez F (2008). PathWays to Doubled Haploidy: Chromosome Doubling During Androgenesis. *Cytogenet. Genome Research* **120**(3-4) 358-369.
Sorger PK, Dobles M, Tournebize R and Hyman AA (1997). Coupling Cell Division and Cell Death to Microtubule Dynamics. *Curr. Opn. in Cell Biology* **9**(6) 807-814.
Stewart ZA, Leach SD and Pietenpol JA (1999). p21Waf1/Cip1 Inhibition of Cyclin E/Cdk2 Activity Prevents Endoreduplication after Mitotic Spindle Disruption. *Mol. Cell. Biology* **19**(1) 205-215.

Sugimoto Shirasu K and Roberts K (2003). Big it up: Endoreduplication and the Control of Cell Size. *Curr. Opn. in Plant Biology* **6**(6) 544-553.

Weingartner M, Criqui MC, Mészáros T, Binarova P, Schmit AC, Helfer A, Derevier A, Erhardt M, Bögre L and Genschik P (2004). Expression of Nondegradable Cyclin B1 Affects Plant Development and Leads to Endomitosis by Inhibiting the Formation of a Phragmoplast. *The Plant Cell* **16**(3) 643–657.