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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

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*Full Length Research Paper*

# Antibacterial activity of *Waltheria indica* Linn (Sterculiaceae), collected from Blouberg area, Limpopo Province, South Africa

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**Mature roots of *Waltheria indica* were collected from Blouberg area, Limpopo Province, republic of South Africa. Water, ethanol and methanol extracts were investigated for antibacterial activity at 5 mg/ml against 10 human pathogenic strains using disc diffusion method. Ethanol extract exhibited potent antibacterial activity against selected strains while methanol extract exhibited largest zone of inhibition of 15.2±2.18 mm against *Bacillus pumilus*. Extracts were further investigated for antibacterial activity using minimum inhibitory concentration (MIC) assay. Lowest MIC of 0.65 mg/ml was exhibited by methanol extract against *Acinetobacter calcaoceuticals anitratus*. Water extract exhibited good MIC of 2.08 mg/ml against *Escherichia coli* while ethanol extract showed lowest MIC of 1.04 mg/ml against *Enterobacter cloacae* and 6.25 mg/ml against *Klebsiella* spp., *Serratia marscens* and *Staphylococcus epidirmidis*. Moreover, ethanol extract possessed a total activity of 433 mL/g against *Enterococcus faecalis*, meaning that the extract can be diluted to 433 mL and would still inhibit growth of these bacteria. These results in a way validate the use of *W. indica* L in the treatment of variety of infections, especially urinary tract infecting bacterial strains.**

**Key words:** *Waltheria indica* L, minimum inhibitory concentration (MIC), disc diffusion, total activity, antibacterial.

## INTRODUCTION

Family Sterculiaceae comprise of species of mainly trees and some herbs indigenous to the tropical rain-forest African region (Sonibare et al., 2009). Several members of this family in Africa have been reported to possess antimicrobial properties, anti-inflammatory activity, high levels of COX-1 inhibition and variety of compounds which includes alkaloids, tannins and cardiac glycosides (Reid et al., 2005; Babalola et al., 2012; Agyare et al., 2012, Hossain et al., 2013, Saradha et al., 2013).

*Waltheria indica*, commonly known as "*Mokhutesela*", is an erect perennial shrublet or a herb of up to ± 500 mm high, stalked leaves with margins shallowly and irregularly toothed (van Wyk and Malan, 1998). Its flowers are yellow and occur in clusters. Globally, its distribution and habitat is mostly in subtropical and tropical zones and in scrub forests, inundated savannas, riverbanks, and sandy

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or clay soils and in disturbed or impoverished soils (Saunders, 2007). Within Blouberg area in Limpopo Province, its roots are used to treat sexually transmitted infections, urinary tract infections, infant illnesses and heart problems. Elsewhere, roots are reported to treat ailments such as diarrhoea, fever, wounds, stomach ache and neurological disorders (Ayantunde et al., 2009; Rodrigues, et al., 2006; Romeiras et al., 2012). The whole plant is reportedly used in the treatment of diarrhoea while leaves are used to treat peptic ulcer (Mathabe et al., 2006; Oluranti et al., 2012). Moreover, sap from the stems of this plant may be used to treat wounds caused by syphilis (Hedimbi and Chinsembu, 2012) while decoction of its leaves combined with those of *Terminalia catappa* may be taken to treat anaemia (Gbadamosi et al., 2012). Phytochemically, a tiliroside compound known as Kaempferol 3-O-β-D-(6"-O-coumaroyl) glucopyranoside has been isolated from this plant (Calderón-Montaño et al., 2011). Moreover, extracts from this plant are reported to possess analgesic, anti-inflammatory and central nervous system depressant activity (Garcia et al., 2010; Mohammed et al., 2007; Hamidu et al., 2008). This paper was aimed at investigating the water, ethanol and methanol extracts from *W. indica* root against nosocomial hospital-acquired bacterial strains which may result in variety of diseases in humans. *Escherichia coli*, *Staphylococcus aureus*, *Enterococcus faecalis*, *Klebsiella spp*, *Staphylococccus epidermidis* and *Enterobacter cloacae* have been frequently isolated from patients with urinary tract infections in tertiary health care facilities world-wide and are some strains were reportedly resistant to gentamycin, penicillin, amoxylin, ciprofloxacin and tetracycline amongst other commonly used drugs in developing countries (Sabir et al., 2014; Drago et al., 2001; Jacobsen et al., 2008; Pallett and Hand, 2010).

## MATERIALS AND METHODS

### Plant materials

Mature roots of *W. indica* L. were collected from William Show farm, Blouberg area, Limpopo Province-Republic of South Africa. Collected specimen were washed with tap water, and then rinsed with distilled water repeatedly until soil debris was removed. Roots were cut into small pieces and dried on the bench. Voucher specimen was collected and identified by National Biodiversity Institute (NBI) in Pretoria, Republic of South Africa.

### Extraction

All the chemicals used including solvents were of AR grade and were obtained from Sigma-Aldrich Co. Ltd. Small pieces of *W. indica* roots were ground into thin powder (2mm mesh size) and extracted (1:3 w/v) twice with boiling water, ethanol and methanol respectively. Organic solvents were evaporated using rotary evaporator, while water extract was freeze dried. Resulting residues were weighed and kept in a refrigerator.

### Selected bacterial strains

A combination of ATCC, clinical isolates and multi-resistant strains were obtained from Department of Biochemistry and Microbiology, University of Zululand. Five Gram negative strains namely *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), *Klebsiella* spp. (317302), *Acinetobacter calcaoceuticals anitratus* (CSIR) and *Serratia marscens* (ATCC 9986) and five Gram positive strains such as *Staphylococcus aureus* (ATCC 6538), *Staphylococcus aureus* (P12702), *Bacillus pumilus* (ATCC 14884) and clinical isolates of *Enterococcus faecalis* and *Staphylococcus epidirmidis* were selected for this study. All organisms were maintained on Mueller Hinton agar plates.

### Antibacterial tests using disc diffusion method

Water, ethanol and methanol extracts from *W. indica* were tested for antibacterial activity by the disc diffusion method according to National Committee for Clinical Laboratory Standard guidelines (NCCLS, 2001). A single colony of the respective organism was aseptically transferred with an inoculating loop to a 15 ml of fresh sterile saline broth in a test tube which was vortexed thoroughly and incubated overnight at 37°C. Turbidity was then adjusted to that of 0.5 McFarland's standard using spectrophotometer (Spec. 20). About 100 µl of the inoculum was aseptically transferred to a labelled disposable Petri-dish containing 15 ml Muller-Hinton agar and spread thoroughly using sterile glass spreader. Sterile paper discs of 5 mm (Mast Disks, UK) were impregnated with 10 µl of 5 mg/ml plant extract dissolved in 5% dimethyl sulfoxide (DMSO) and gently placed individually on the seeded agar. Plates were allowed to dry for one hour and later incubated in an inverted position at 37°C in over night.

Zones of inhibition were measured using a ruler, including the diameter of sterile paper disc. Streptomycin and penicillin (10 µg/disc) were used as positive controls. Negative controls were performed using paper discs loaded with 10 µl of 5 % DMSO (Merck, RSA). Each experiment was repeated.

### Minimal inhibitory concentrations (MIC) using micro dilution assay

Extracts showing activity in Disc Diffusion were chosen to assay the minimal inhibitory concentration (Eloff, 1998) using the micro plate broth dilution with slight modification. The 24 h old culture was diluted 1:100 with freshly prepared Muller-Hinton broth. About 100 µl of extracts (50 mg/ml in 5% DMSO) were added to multi well plate containing 100 µl of freshly prepared broth and serially diluted, yielding 12.5 mg/ml in the first well. Plates were then incubated over night at 37°C. About 40 µl of 2 mg/ml freshly prepared iodo-nitro-tetrazolium chloride were added to each well and incubated for 1 h at the same temperature. Streptomycin sulphate was used as control. The MIC was defined as the lowest concentration of the extract to inhibit bacterial growth. To compare the activity of different extracts, the total activity in mL/g was calculated by dividing the total mass in mg extracted from 1 g of plant material by the MIC value in mg/ml (Eloff, 2000).

## RESULTS AND DISCUSSION

Due to higher resistance of microorganisms to synthetic drugs, there is a need to screen medicinal plants for antibacterial activity, a first step towards finding the proper substitutes. Nosocomial infections increase the cost of medical care, extend hospital stay and reflect on

**Table 1.** Antibacterial activity of extracts from *Waltheria indica* Linn.

| Bacteria | Water | Ethanol | Methanol | Streptomycin 10 µg | Penicillin 10 µg |
|---|---|---|---|---|---|
| *E. coli* | 13.6 ± 0.38 | 11.1 ± 0.18 | 10.1 ± 0.85 | 27.0 ± 0.0 | 12.7 ± 0.88 |
| *E. cloacae* | R | 10.0 ± 0.0 | 10.0 ± 0.0 | 16.7 ± 0.88 | 8.7 ± 0.33 |
| *Klebsiella spp.* | 9.9 ± 0.57 | 12.7 ± 0.41 | 12.0 ± 0.0 | 16.0 ± 0.67 | 16.3 ± 0.67 |
| *S. marcescens* | R | 9.9 ± 0.57 | 9.9 ± 0.57 | 17.3 ± 0.33 | 11.3 ± 0.33 |
| *Acinetobacter calcaoceuticals anitratus* | 11.7 ± 1.32 | 10.0 ± 0.0 | 10.0 ± 0.0 | 22.0 ± 0.0 | 12.0 ± 0.0 |
| *S. aureus* | R | 12.2 ± 0.67 | R | 17.7 ± 1.53 | 11.3 ± 0.33 |
| *S. aureus* (P12702) | 9.9 ± 0.57 | 11.7 ± 0.88 | R | 24.3 ± 1.16 | 16.3 ± 0.67 |
| *B. pumilus* | 12.6 ± 1.06 | 11.7 ± 0.88 | 15.2 ± 2.18 | 18.7 ± 0.67 | 11.3 ± 0.88 |
| *E. faecalis* | 9.9 ± 0.57 | 10.5 ± 0.82 | 13.5 ± 0.71 | 12.7 ± 1.16 | 11.7 ± 1.52 |
| *S. epidirmidis* | R | 11.0 ± 1.26 | R | 18.3 ± 0.33 | 14.3 ± 0.67 |

R, resistant. Zones of inhibition (mm) were reported as mean of three replicates ± SEM.

the morbidity and mortality of the admitted patients (Melaku et al., 2012). Results for antibacterial activity of three extracts of *Waltheria indica* are shown in Table 1. Methanol extract showed activity against all selected gram negative bacterial strains, with zones of inhibition ranging from 9.9±0.57 mm (*S. marcescens*) to 12.0±0.00 mm (*Klebsiella* spp.), hence narrow spectrum. Besides being involved in neonatal sepsis and causing bacterial pneumoniae in adults, *Klebsiella* spp. may primarily attack immunocompromised patients mostly suffering from mellitus diabetes and chronic pulmonary infections resulting in various fatalities if untreated (Podschun and Ullmann, 1999), while *S. marcescens* is known to produce different enzymes including chitinase and lipase resulting in devastating bacteremia, respiratory tract infections, meningitis and infective endocardia amongst other infections if not treated (Hejazi and Falkiner, 1997).

Ethanol extract exhibited largest zone of inhibition of 15.2±2.18 mm against *Bacillus pumilus*, which is greater than that of penicillin (11.3±0.80 mm) against similar organism. Moreover, it exhibited activity against all Gram positive and Gram negative strains, hence broad spectrum. Similar trend has been reported elsewhere (Mukhtar and Ghori, 2012). Besides exhibiting good DPPH free radical scavenging activity, ethanol extract of leaves this plant reportedly exhibited activity against *B. subtilis* and *E. coli*, with zones of inhibitions ranging from 7 to 25 mm (Garba et al., 2012). Else where, 95 % ethanol extract of this plant exhibited activity of 13 mm (zone of inhibition) against *Escherichia coli*, *Staphylococcus aureus* and *Enterobacter aerogens* (Olajuyigbe et al., 2011). Elsewhere, the *n*-hexane extract from shoots of this plant exhibited MIC of 500 µg/ml against *S. aureus* (Maregesi et al., 2008). It is difficult to compare these results with the current study because of differences in nature and types of bacteria used, locality or geographical area, plant part used and solvent type.

Although water extract was the least active, it exhibited activity of 13.6±0.38 mm against *E. coli,* 12.6±1.04 mm against *B. pumilus* and 11.7±1.32 mm against *A. calcaoceuticals anitratus*. *E. coli* is by far the most common cause of nosocomial or urinary tract infection among hospitalised patients (Shilpi et al., 2012; Wilson and Gaido, 2004). Moreover, it may produce plasmid-mediated AmpC β-lactamases (PABLs) which may be difficult to detect and might interfere with infection control processes (Lee et al., 2009). Elsewhere, aqueous extract was reported to possess trypanocidal activity against *Trypanosoma brucei brucei* (Bala et al., 2009).

*S. marscens* and *E. cloacae* were amongst four strains found resistant to water extract. These organisms may become resistant to variety of cephalosporins and monobactams by overproducing their chromosomal AmpC β-lactamases (Pitout et al., 2010). *S. aureus* and *S. epidirmidis* were resistant to both water and methanol extract. These organisms are reported to be the most common causes of medical-device associated infections, including septicemic loosenings of orthopaedic implants (Krimmer et al., 1999). Selected strains were more susceptible to streptomycin than penicillin.

MIC values of *W. indica* are recorded in Table 2. Although water extract did not show good inhibition against selected strains, it exhibited MIC of 2.08 mg/ml against *E. coli,* while methanol extract exhibited MIC of 4.17 mg/ml against *E. cloacae, Klebsiella spp.* and *S. aureus.* Furthermore, Ethanol extract exhibited MIC between 1.04 (*E. faecalis*) and 6.25mg/ml against *Klebsiella spp*, *S. marscens* and *S. epidirmidis*. All the selected strains were susceptible to streptomycin sulphate.

Total activities were calculated to validate the quality of the extracts tested in Table 2. Ethanol extract exhibited highest total activity of 433 mL/g against *Enterococcus faecalis*. Compared to water and methanol extracts, ethanol extract exhibited potent total activity against selected bacterial strains. According to Makhafola and Eloff (2012), total activity determines quality of an extract (how much can an extract be diluted and still kill bacteria). However, it is dependent upon quantity of the

**Table 2.** Minimal Inhibitory concentrations (MIC) of extracts from *W. indica* (mg/ml) and total activity (mL/g) of *Waltheria indica* extracts

| Bacteria | Water | Ethanol | Methanol | Streptomycin sulphate |
|---|---|---|---|---|
| **Minimal Inhibitory concentrations (MIC) of extracts from *W. indica* (mg/ml)** | | | | |
| *E. coli* | 2.08 | 3.65 | 6.25 | 0.04 |
| *E. cloacae* | 6.25 | 4.12 | 4.17 | 0.32 |
| *Klebsiella spp.* | 12.5 | 6.25 | 4.17 | 0.04 |
| *S. marcescens* | 4.17 | 6.25 | 12.5 | 0.32 |
| *Acinetobacter calcaoceuticals anitratus* | 4.17 | 1.90 | 0.65 | 0.08 |
| *S. aureus* | 6.25 | 5.21 | 4.17 | 0.08 |
| *S. aureus* (P12702) | 6.25 | 5.21 | 12.5 | 0.32 |
| *B. pumilus* | 6.25 | 2.08 | 3.65 | 0.08 |
| *E. faecalis* | 12.5 | 1.04 | 1.30 | 0.03 |
| *S. epidirmidis* | 2.60 | 6.25 | 12.5 | 0.08 |
| **Total activity (mL/g) of *Waltheria indica* extracts** | | | | |
| *E. coli* | 39 | 123 | 22 | |
| *E. cloacae* | 13 | 109 | 33 | |
| *Klebsiella* spp. | 6.5 | 72 | 33 | |
| *S. marcescens* | 20 | 72 | 11 | |
| *Acinetobacter calcaoceuticals anitratus* | 13 | 236 | 212 | |
| *S. aureus* | 6.5 | 86 | 33 | |
| *S. aureus* (P12702) | 13 | 86 | 11 | |
| *B. pumilus* | 13 | 216 | 38 | |
| *E. faecalis* | 13 | 433 | 106 | |
| *S. epidirmidis* | 31 | 72 | 11 | |

plant material extracted from dried plant material and MIC of extract. Earlier, the methanol extract of the root was reported active against *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Proteus vulgaris* revealing 3.13, 0.65 and 2.08 mg/ml respectively while ethanol extract exhibited minimum bactericidal concentration of 2.08 and 4.17 mg/ml against *P. vulgaris* and *P. aeruginosa* respectively (Mongalo et al., 2012). In the current study, the clinical isolate of *E. faecalis* was susceptible to all the selected extracts with MIC ranging from 1.04 to 12.5 mg/ml. The MIC of 3 mg/ml is referred to as most potent (Mongalo et al., 2013; Aliyu et al., 2008), thereby validating the potency of methanol and ethanol extracts which are thus the most potential candidates for bioassays in trying to find new therapeutic compounds used to treat human infections which may be resistant to current antibiotics. These results and the currently reported data, validates the ethnomedicinal use of *W. indica* root in the treatment of variety of infections, especially urinary tract infections as majority of these organisms are the major causative agents of such infections.

Besides various fractions showing activity against *E. coli*, *P. aeruginosa* and *Salmonella typhi*, aqueous extract of the root revealed the presence of tannins, saponins, steroids and Cardiac glycosides (Zailani et al., 2010). Tannins may prevent development of microorganisms by precipitating microbial protein and making nutritional proteins unavailable (Prasad et al., 2008) and may hasten the healing of wounds and inflamed mucous membrane (Njoku and Akumefula, 2007). Saponins have detergent properties and serve as lytic agents and exhibit anti-inflammatory properties (Abukakar et al., 2008) while cardiac glycosides are known to work by inhibiting the ($Na^+/K^+$) pump, thereby increasing the amount of $Ca^{2+}$ ions available for the contraction of heart muscles which improves cardiac output and reduces distensions of heart, thus used in the treatment of congestive heart failure and cardiac arrhythmia (Ngbede et al., 2008).

## Conclusions

Ethanol extract from *W. indica* exhibited potent anti-bacterial activity against selected bacterial strains. In a way, this work validates the use of *W. indica* in the treatment of nosocomial infections. However, there is a need to investigate the antimicrobial activity of this plant against microbes belonging to the traditional sphere of sexually transmitted infections. Biological activity exhibited by ethanol and methanol extracts in this work makes them the potential candidates for individual compounds isolation. Such compounds should also be investigated for various biological activities.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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*Full Length Research Paper*

# Cloning and characterization of pre-miR159a and pre-miR1123 from Indian hexaploid wheat and their evolutionary linkage analysis

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**MicroRNAs (miRNAs) are important post-transcriptional regulator of genes in plants. In spite of their unusual small size (-21-24 nt), the evolutionary history of miRNA gene families appears to be similar to their protein-coding counterparts. Although many miRNA genes are conserved across the plant species, the same gene family varies significantly in size and genomic organization in different species. In the present study, we characterized *Tae* pre-miR159a (*Triticum aestivum* precursor-miR159a) and *Tae* pre-miR1123 from a most popular Indian wheat cultivar Agra local. miR159a is conserved across the species while miR1123 is reported in wheat. Sequence analysis of the pre-miR159a among different plant species suggest conserved motif in duplex miRNAs. Beside mature miRNAs, certain other regions are also found to be conserved across species which might be the site of processing of the precursors by DCL1. Sequence identity matrix suggests 43-82% variation in precursor of *Tae* AL pre-miR159a (*Tae* Agra local pre-miR159a) across the species. On the other hand, *Tae* AL pre-miR1123 (*Tae* Agra local pre-miR1123) showed >83% sequence similarity with the reported sequence of *Tae* pre-miR1123. Sequence similarity of precursor and matured miRNA across the species suggests the common evolutionary point from the same gene family which is essential for the processing and stem loop structure of the precursor miRNAs.**

**Key words:** Cloning, stem-loop structure, sequence comparison, duplex, precursor.

## INTRODUCTION

MicroRNAs are generated from larger precursors which usually form a stem-loop structure (Li and Mao, 2007). In plants, a ribonuclease III-like protein in the nucleus called DICER-LIKE 1 (DCL1) is responsible for processing of

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the primary miRNA (pri-miRNA) gene transcript (Papp et al., 2003). The processed pri-miRNA is called pre-miRNA which is eventually processed into mature miRNA: miRNA* duplex (guide stand: passenger strand) (Bartel and Chen, 2004). The mature miRNAs duplex is then transported into the RNA-induced silencing complex (RISC) which either degrade the corresponding target mRNA or repress its translation. In both animal and plant genomes, multiple precursors are present to produce similar mature miRNA (Tanzer et al., 2005; Maher et al., 2006). Animal genomes have a large number of small miRNA gene families while plant genomes have fewer but larger miRNA gene families. Plant miRNAs derived from the same gene family are often highly similar unlike animal miRNA genes where divergence has occurred even on the mature miRNA sequences. The similarities are not only restricted to mature miRNA regions but also throughout the genes suggesting the recent origin of plant miRNA gene families which might still be going on. Recently Zhang et al. (2006) has reported a total of 481 miRNAs from more than 6 million plant EST sequences that belong to 37 miRNA families from 71 different plant species.

In the recent past, efforts have been put forth to identify the different mechanism of evolutionary origin of miRNA genes. Four mechanisms have been proposed so far. First mechanism implies evolutionary origin of miRNA genes *via* duplication of protein coding genes (Fahlgren et al., 2007, 2010). Second mechanism involves the conversion of transposable elements in to miRNA genes (Piriyapongsa and Jordan, 2008). Third and fourth includes duplication of pre-existing miRNA genes with subsequent mutation and natural origin by spontaneous mutation from hairpin structure in the genome respectively (Li and Mao, 2007; De Felippes et al., 2008). Plant miRNAs have been reported to be conserved across wide taxa of gymnosperms, fern, moss, and liverwort (Floyd and Bowman, 2004; Cuperus et al., 2011). Conserved nature of miRNAs across the species might be due to the specific mechanism of origin over the years.

MicroRNAs are a gene family united not by a common sequence, nor by a common phenotypic output, but by a unique mode of biogenesis and molecular mechanism (Axtell, 2008). Most of the predicted targets of these microRNAs are members of transcription factor “gene families” involved in developmental patterning or cell differentiation (Rhoades et al., 2002) that is, miRNA genes also form gene families. Since the evolution of animal miRNA gene families has been a subject of many paper (Tanzer and Stadler, 2006; Bompfunewerer et al., 2005), in this paper we cloned and characterized the *Tae* pre-miR159a and *Tae* pre-miR1123 from Indian hexaploid wheat cultivar Agra local and compared the obtained sequences with the sequences of same miRNA reported in the other plant species to study the evolutionary linkages of miRNA gene families in wheat genomes.

## MATERIALS AND METHODS

### Plant materials

Hexaploid wheat cultivar Agra Local was grown at 30 seeds per 10 cm pot at 15-25°C temperature under natural lighting conditions for ten days in glass house at Division of Plant Pathology, IARI, New Delhi. This cultivar is characterized by its larger area of production as well as susceptibility to stem rust as reported by our previous publication (Gupta et al., 2012). Leaf samples were collected and quickly frozen in liquid nitrogen and stored at -800°C until used for total RNA isolation and subsequent cloning.

### Isolation of total RNA from leaf sample

Leaf samples were harvested from healthy plants of 10 days old seedlings and quickly frozen in liquid nitrogen and stored at -80°C prior to total RNA isolation. Total RNA was extracted from 100 mg of leaf tissues using RNeasy Plant Kit (Qiagen) according to manufacturer’s instruction. The purity and concentration of RNA was determined by spectrophotometer NanoDrop, ND-1000 (NanoDrop technologies).

### cDNA synthesis and PCR amplification

Specific primers were synthesized from Sigma Pvt Ltd. Primer sequences of pre-miR159a were designed from chromosome no. 1 of *Arabidopsis* collected from NCBI and pre-miR1123 were designed from wheat miR1123 sequences available in Sanger miRBase http://miRNA.sanger.ac.ck. For cDNA synthesis, Reverse transcriptase (RT) (NEB) was used. cDNA was synthesized using the reverse primer of both the miRNAs (pre-miR159a reverse primer: 5’TGA GTC GAC ATG TAG AGC TCC CTT CAA TCC3’ Tm:90°C; pre-miRNA 1123 reverse primer: 5’TTC TAT GAG ACC AGG TCT CAC3’ Tm:62°C) and the reaction mixture was prepared in 200 µl microfuge tube.

The reaction was carried out at 42°C for 60 min followed by purification by cDNA purification kit (Qiagen). A ~175 bp and ~220 bp fragment containing the entire sequence of the wheat pre-miR159a and miR1123 respectively were amplified by PCR amplification using complementary DNA (cDNA). 20 µl PCR reaction volume was prepared using 2 µl of cDNA (25 ng/µl) as template, 1 µl (10 mM) reverse primer, 1 µl (10 mM) forward primer (pre-miR159a forward primer: 5’CAC CAC AGT TTG CTT ATG TCG GAT CC3’ Tm:78°C; pre-miRNA 1123 forward primer: 5’AAA ATT ATA TGA GAC CAG GCT C3’ Tm:60°C), 1 µl (25mM) $MgCl_2$, 0.25 U Taq DNA polymerase (NEB) and 11.5 µl sterile distilled water (SDW) using PCR programme (94 °C for 4 min followed by 30 cycles of 94°C for 40 s, 58°C for 30 s and 72 °C for 90 s for pre-miR159a and 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 58°C for 1 min and 72°C for 1 min for pre-miR1123). PCR amplification was performed in a thermo cycler separately (ERICOMP Power Block II System). The amplified product was separated on 1% agarose gel run in 1X TAE buffer and detected by ethidium bromide staining.

### Cloning of amplified product and restriction analysis

Amplified products were gel eluted and purified by Genei PCR

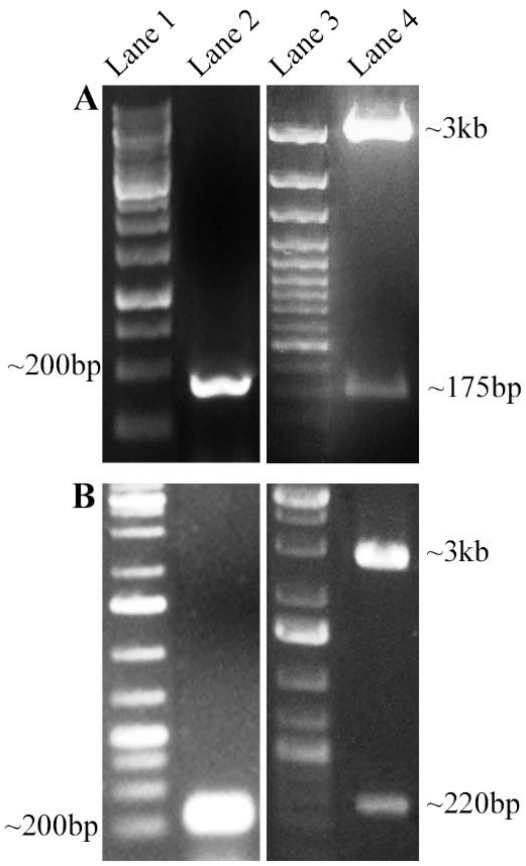


**Figure 1.** Amplification and confirmation of pre-miR159a and pre-miR1123 from Agra local wheat cultivar. **(A)** Lane 1 and 3, molecular weight marker (1 kb and100 bp respectively); lane 2, PCR amplicon of pre-miR159a; lane 4, restriction digestion of pre-miR159a to release ~175 bp amplicon from pGEMT. **(B)** Lane 1 and 3, molecular weight marker (1 kb); lane 2, PCR amplicon of pre-miR1123; lane 4, restriction digestion of pre-miR1123 to release ~220 bp amplicon from pGEMT.

purification kit following the manufacturer's protocol. Purified PCR product was used for T-A cloning in p-GEMT Easy vector (3015 bp) provided by Promega according to Sambrook and Russell (2001). Positives clones were selected based on blue white screening followed by colony PCR and *Not I* restriction digestion. After restriction analysis, positive clones were sequenced and obtained sequences were analysed *in silico* using BioEdit Sequence Alignment Editor (version 5.0.9).

#### Bioinformatic analysis

Raw sequences of both the pre-miRNAs obtained after sequencing were used to analyse the evolutionary linkage with the sequence of same pre-miRNAs across the different plant species. Pre-miRNA159a sequences from different plant species were collected from Sanger miRBase (http://miRNA.sanger.ac.ck). The sequence of wheat specific Pre-miR1123 from Agra local cultivar was compared with the reported sequence of wheat. BioEdit Sequence Alignment Editor (version 5.0.9) was used for the sequence and multiple sequence alignment analysis. Secondary stem loop structure of both the pre-miRNA sequences was done using MFOLDROOT secondary structure prediction software (http://www.bioinfo.rpi.edu/applications/mfold).

## RESULTS

### Amplification, cloning and sequencing of pre-miR159a

An amplicon of ~175 bp corresponding to *Tae* AL pre-miR159a was obtained by PCR from wheat cultivar Agra local (AL). The PCR purified amplicon (~175 bp) was then cloned in p-GEMT Easy vector (Figure 1A, lane 2). Fifty white colonies were found on X-gal, IPTG, ampicillin plate. All these colonies were streaked on master plate separately. From the master plates, ten colonies were subjected to colony PCR using the specific primer described earlier. On the basis of the colony PCR results, five clones were selected for recombinant plasmid isolation. Final confirmation was carried out by releasing ~175 bp fragment using *Not I* restriction digestion (Figure 1A). Two clones (p-GEMT- *Tae* AL pre-miR159a) was sequenced to get the nucleotide sequence of AL pre-miR159a. Similar approach was used to amplify and clone the ~220 bp amplicon of AL pre-miR1123 (Figure 1B, lane 4). The raw sequences obtained were analysed using BioEdit Sequence Alignment Editor (version 5.0.9) to get the precursor sequence of *Tae* AL pre-miR159a and *Tae* AL pre-miR1123.

### Sequence analysis of pre-miR159a and pre-miR1123

Sequence analysis of pre-miR159a from 11 different reported plant species indicates the presence of exact 21 nt long mature miRNA towards the 3' end. The passenger strand of mature miRNA residing at 5' end of gene showed 5-6 mismatches. On close comparison, it was found that mismatches in miRNA duplex are conserved in the species (Figure 2). Although the overall sequence similarity of *Tae* AL pre-miR159a was 82% with *At* pre-miR159a (*At*: *A. thaliana*) and only 64% with reported *Tae* line3338 pre-miR159a, the pattern of mismatches of duplex miRNA (miRNA:miRNA*) within species were found to be exactly same (Figure 3A). Sequence identity matrix suggests 43-82% variation in precursor of miR159a (Table 1). Multiple sequence alignment showed conserved motif at 3' and 5' end of miRNA gene representing mature miRNA and its complementary sequence respectively. Besides this, certain motif were found to be conserved which might be essential for processing of precursor sequences by DCL1.


**Figure 2.** Comparison of matches and mismatches in pre-miR159a duplex across the species. Extreme left represents the name of species along with miRNA with accession number while mature miRNAs are represented in between the loop structure (red color). Pre-miR159a in three species namely *G. max, Z. mays* and *P. vulgaris* have two mature miRNA sequences in the respective backbone which is denoted by green colour. Mature miR159a.1 of *P. vulgaris* and miR159a.3p of *G. max* and *Z. mays* were used to compare with mature miRNAs of other species

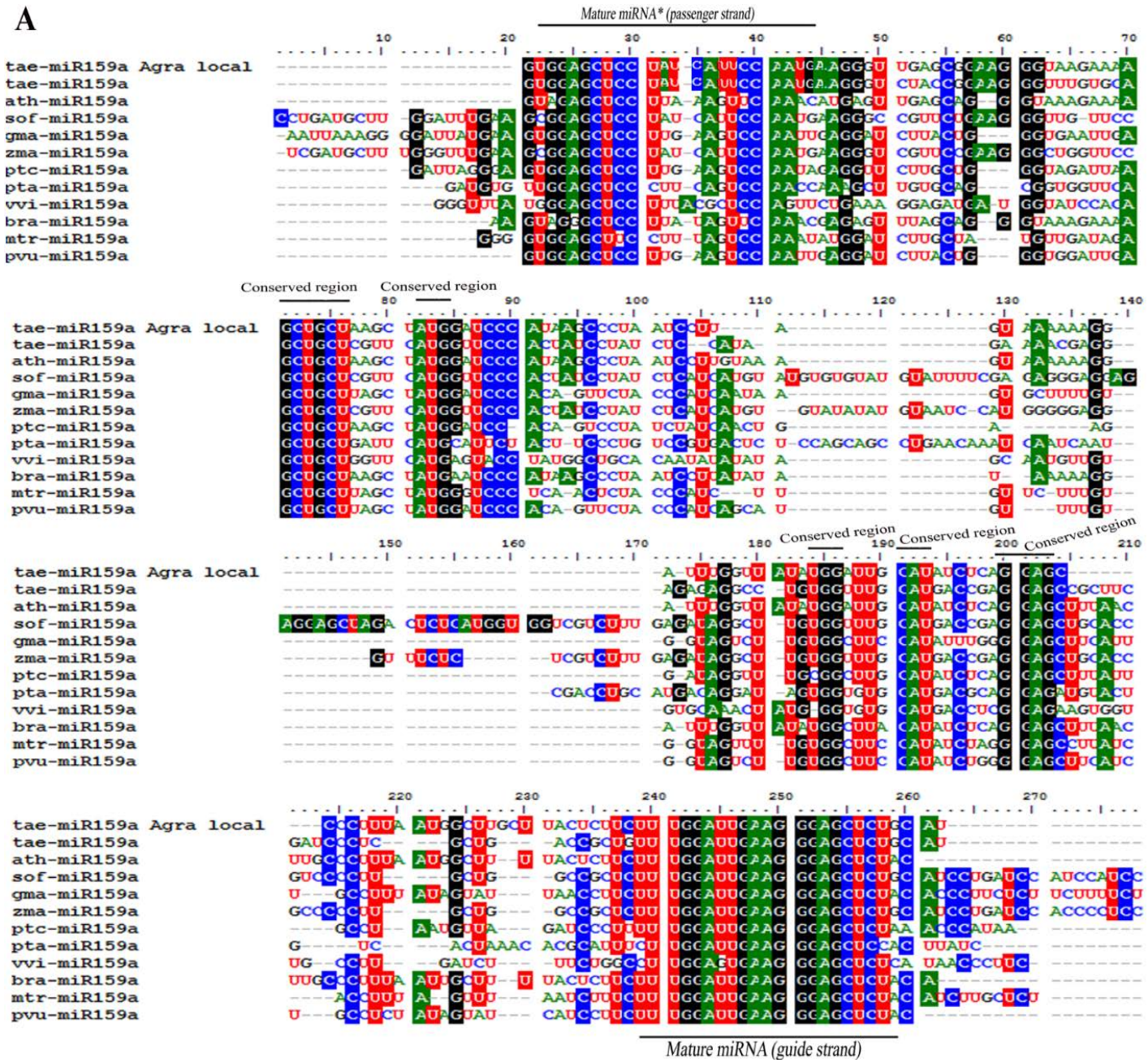


**Figure 3. A.** Comparision of pre-miR159a sequence isolated from Agra local wheat cultivar with different species. Mature miRNAs are conserved in nature across the species while the sequences flanking to the mature miRNA are not conserved across the backbone length. In between the backbone conserved nucleotides are highlighted. (tae, *T. aestivum*; ath, *A. thaliana*; sof, *S. officinarum*; gma, *G. max*; zma, *Z. mays*; ptc, *P. trichocarpa*; pta, *P. taeda*; vvi, *V. vinifera*; bra, *B. rapa*; mtr, *M. truncatula*; pvu, *P. vulgaris*). **B.** Comparison of pre-miRNA1123 sequence isolated from Agra local (*Tae* AL) wheat cultivar with reported sequences of line *Tae* 3338.

Phylogenetic relationship suggests that *Tae* AL pre-miR159a is showing close relationship with backbone (pre-miRNA) of *Arabidopsis, Brassica, and Triticum*. The *Tae* line 3338 pre-miR1123 sequence was downloaded from miRNAs Registry database (http://miRNA.sanger.ac.uk) and used to compare with the *Tae* AL pre-miR1123. On comparing, *Tae* AL pre-miR1123 showed >83% sequence similarity with the reported sequence of pre-miR1123 from *Tae* line 3338 (Figure 3B).

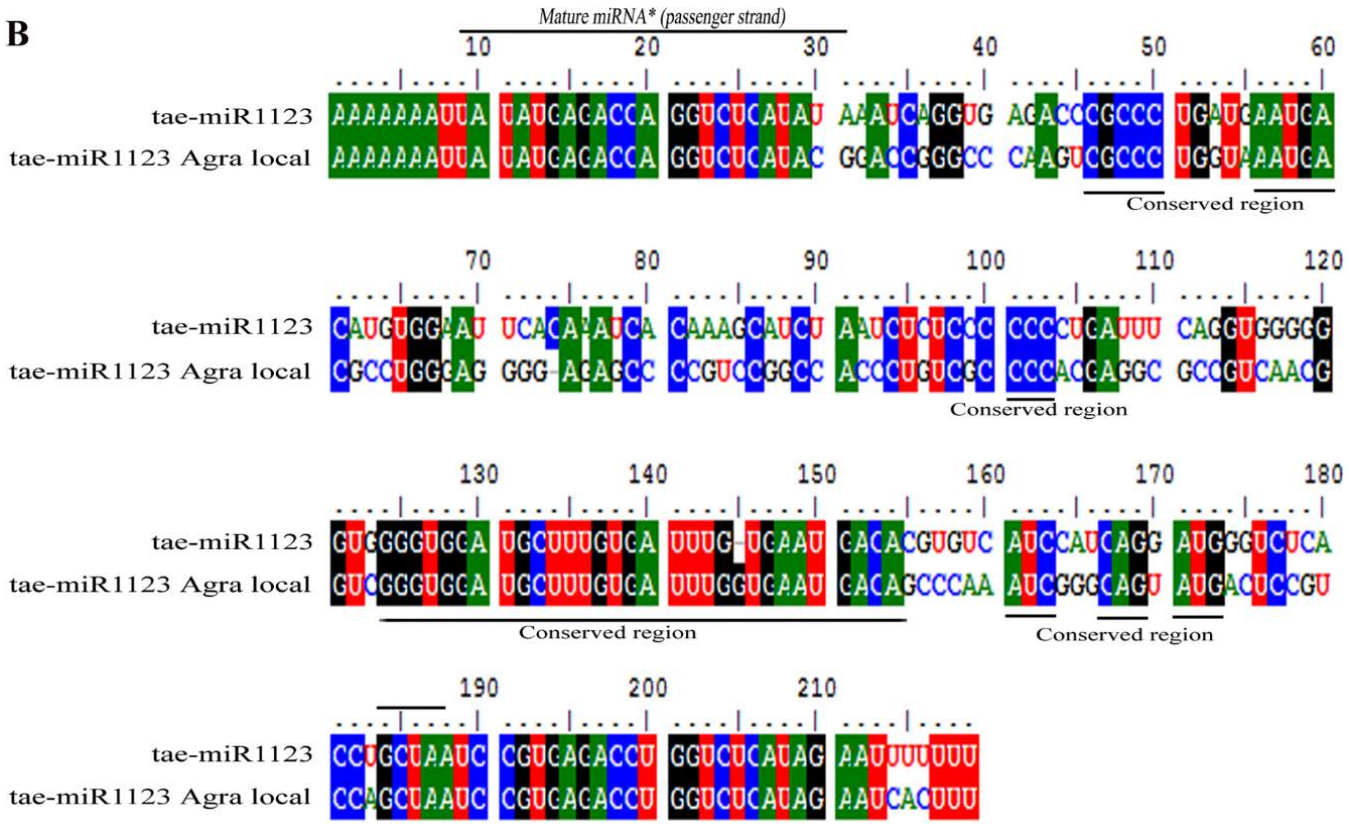


**Figure 3.** Contd.

**Table 1.** Sequence identity matrix of pre-miR159a.

| Parameter | tae | tae Agra local | ath | sof | gma | zma | ptc | pta | vvi | bra | mtr | pvu |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **tae** | ID | | | | | | | | | | | |
| **tae Agra local** | 0.642 | ID | | | | | | | | | | |
| **ath** | 0.566 | 0.826 | ID | | | | | | | | | |
| **sof** | 0.575 | 0.434 | 0.394 | ID | | | | | | | | |
| **gma** | 0.454 | 0.515 | 0.524 | 0.443 | ID | | | | | | | |
| **zma** | 0.613 | 0.463 | 0.419 | 0.846 | 0.474 | ID | | | | | | |
| **ptc** | 0.5 | 0.559 | 0.562 | 0.418 | 0.672 | 0.45 | ID | | | | | |
| **pta** | 0.479 | 0.43 | 0.43 | 0.418 | 0.395 | 0.472 | 0.466 | ID | | | | |
| **vvi** | 0.479 | 0.434 | 0.433 | 0.382 | 0.434 | 0.396 | 0.42 | 0.415 | ID | | | |
| **bra** | 0.549 | 0.776 | 0.909 | 0.394 | 0.533 | 0.419 | 0.592 | 0.417 | 0.418 | ID | | |
| **mtr** | 0.505 | 0.564 | 0.563 | 0.403 | 0.686 | 0.43 | 0.639 | 0.45 | 0.465 | 0.558 | ID | |
| **pvu** | 0.545 | 0.59 | 0.619 | 0.397 | 0.771 | 0.422 | 0.701 | 0.433 | 0.45 | 0.607 | 0.751 | ID |

### *In silico* prediction of secondary loop structure of pre-miR159a and pre-miR1123

The sequences of *Tae* line3338 pre-miR159a and *Tae* line 3338 pre-miR1123 were downloaded from miRNAs Registry database (http://miRNA.sanger.ac.uk) and used to predict the secondary stem loop structure using MFOLDROOT secondary structure prediction software

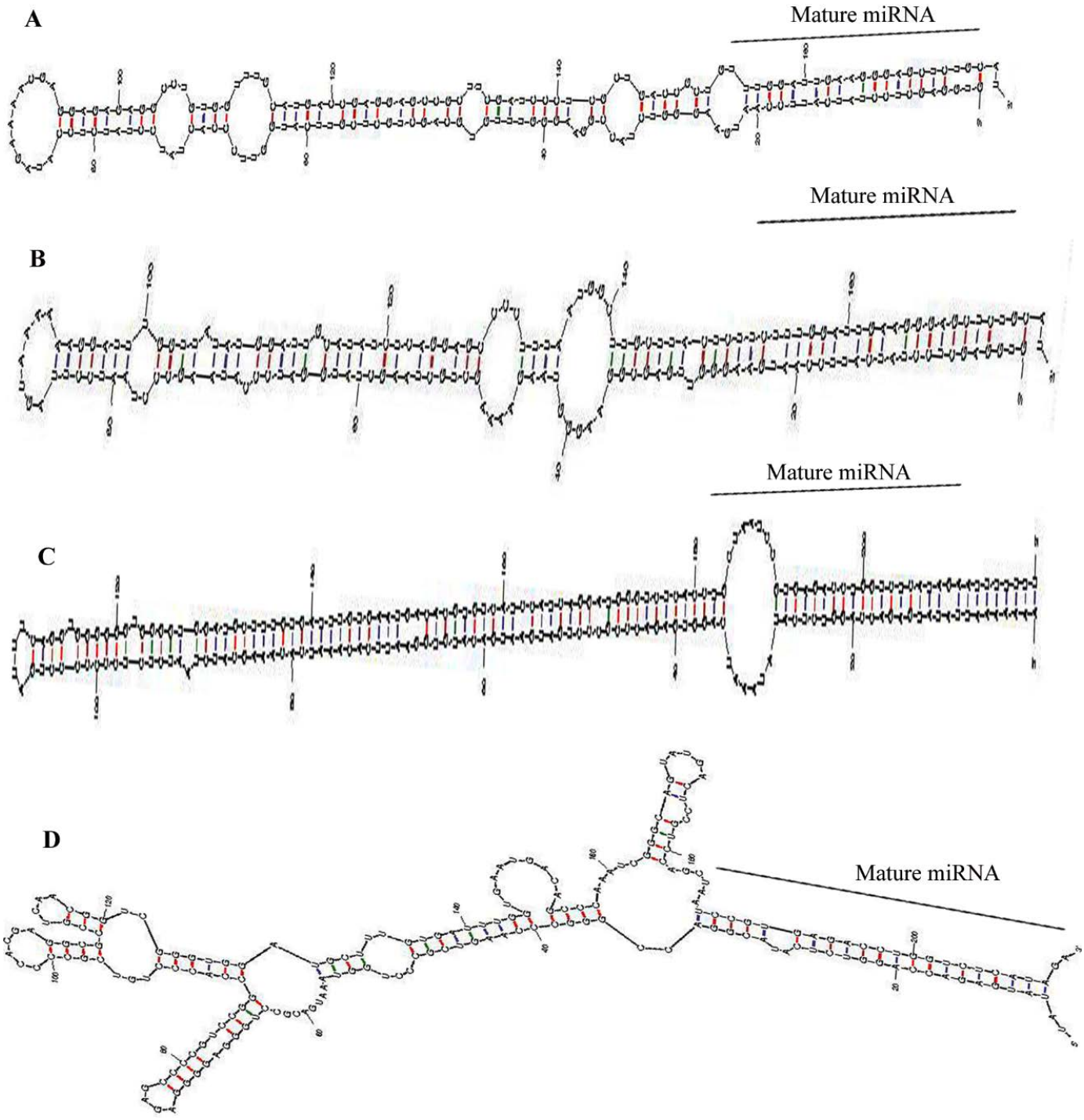


**Figure 4.** Comparison of secondary stem loop structure of pre-miR159a and pre-miR1123 in wheat. (A) Stem loop structure from reported line *Tae* 3338. (B) Stem loop structure from Agra local wheat cultivar (*Tae* AL). (C) Stem loop structure from reported line *Tae* 3338 (D) Stem loop structure from Agra local wheat cultivar (*Tae* AL).

(http://www.bioinfo.rpi.edu/applications/mfold).

Comparison of secondary structure of *Tae* line 3338 pre-miR159a with *Tae* AL pre-miR159a is shown in Figure 4A ans B and that of *Tae* line 3338 pre-miR1123 with *Tae* AL pre-miR1123 in Figure 4C and D. This secondary loop structure indicates the presence of conserved mature miRNA at 3' end and various other conserved motifs throughout the backbone. These conserved motifs might

be playing important role in processing.

## DISCUSSION

To date, limited evidence is available about the origination of miRNA genes in plant genomes. The characteristic stem loop structure and the functional mode by which miRNAs pair with their target genes support the hypothesis that the *de novo* generation of miRNA genes is related to their target genes (Allen et al., 2004). Sequence divergence in miRNA backbone among different plant species maintain with the fold back structure and recognition by DCL1. Among various plant species the sequence divergence continued until the point that the miRNA:miRNA* forming a duplex were maintained. Till date no specific model has been proposed on sequence similarity between miRNA genes. In plants, miRNA gene of same family are often scattered throughout the genome indicating the shuffling since the amplification of these families.

Mature miR159a is found to be nearly conserved across the species although the passenger strand showed 30-35% variability. Mature miR1123 sequences are 100% conserved in two genotypes of the same species. The duplex miRNA strands (guide: passenger) in the stem loop structure of pre-miRNA showed a similar pattern within the species which differs across species. The overall sequence identity matrix showed variation from 43-82% with no such species specific consideration. These results suggest that sequence variation in the backbone maintains two important features: (1) mature miRNA fold back structure and (2) sequence required for its processing.

### Conflict of Interests

The author(s) have not declared any conflict of interests.

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*Full Length Research Paper*

# Molecular characterization and diversity analysis in chilli pepper using simple sequence repeats (SSR) markers

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**India is considered to be the secondary center of diversity of chilli pepper, especially of *Capsicum annuum*. Simple sequence repeats (SSRs) are the most widely used marker system for plant variety characterization and diversity analysis especially in cultivated species which have low levels of polymorphism. The diversity analysis of 64 chilli pepper accessions, mostly of Indian origin, was performed using 50 SSR markers. Twenty seven (27) polymorphic primers amplified a total of 75 alleles with an average of 2.78 alleles per locus. Maximum of four alleles were amplified by the primer AVRDC PP 32. The polymorphic information content (PIC) values ranged from 0.39 (AVRDC PP 138) to 0.78 (AVRDC PP 18), with an average of 0.59. Based on the PIC values, primers AVRDC PP 18 was found to be the most informative (0.78), followed by the primers AVRDC PP 32 (0.69) and AVRDC PP 03 (0.66). Using the given set of primers, it was possible to characterize all but two pairs of accessions from each other. The analysis allowed grouping of the test germplasm into nine clusters. Based on diversity analysis, genotypes were identified for developing mapping populations, produce heterotic $F_1$ hybrids and attempt crosses for genetic improvement of the crop.**

**Key words:** Capsicum, genetic diversity, molecular characterization, simple sequence repeats (SSR) markers.

## INTRODUCTION

Chilli pepper (*Capsicum annuum* L.) (Solanaceae) has a chromosome number 2n=2x=24. It is indigenous to South America and was first introduced in India from Brazil by Portuguese towards the end of 15$^{th}$ century (Basu and Krishna, 2003). Pepper is an often cross pollinated crop and, therefore, exhibits wide variability for different qualitative and quantitative traits (Tanksley, 1984). There are five cultivated species of peppers including *Capsicum annuum* L, *C. frutescens*, *C. chinense*, *C. pubescens* and *C. baccatum* (Heiser and Smith, 1957). India is considered to be the secondary center of diversity for chilli (IBPGR, 1983), especially of *C. annuum*, the most

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**Abbreviations: AVRDC,** Asian Vegetable Research and Development Center; **DUS,** distinctness, uniformity and stability; **IBPGR,** International Board for Plant Genetic Resources; **UPGMA,** unweighted pair group method with the arithmetic averaging; **PIC,** polymorphism information content.

important cultivated species. North-Eastern states are home to the genetic variability where several interspecific hybrids/derivatives were originated, among which Naga King is one of the world's hottest peppers. Over the years, chilli has become an important commercial crop of India and the country is currently the leading producer, consumer and exporter of peppers in the world. Although chilli pepper is cultivated almost throughout the country, Andhra Pradesh alone accounts for 25% of the total area and 40-50% of the total national production. In the world trade, India contributes about 25% of the total global pepper exports (Anonymous, 2011).

Genetic resources are the most valuable and essential basic raw material to meet the current and future needs for genetic improvement of any crop. Characterization of the germplasm is important for its identification and registration with the competent authority for plant variety protection. Conventionally, morphological markers called descriptors were used for varietal identification and genetic diversity analysis in plants that demands collection of extensive data at different locations. However, the level of polymorphism for morphological characteristics in elite germplasm is sometimes too limited and inadequate to allow for variety/genotype discrimination (Geleta et al., 2005). The traditional method of distinctness, uniformity and stability (DUS) testing is time-consuming and expensive, requiring large areas of land and skilled personnel, and is often subjective due to environmental influences (Singh et al., 2004). Further, taxonomy of the genus *Capsicum* is confusing and sometimes it is difficult to identify an accession using only subjective morpho-agronomic data (Costa et al., 2006).

In the last decade or so, molecular markers such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs), and simple sequence repeats (SSRs) have been developed for pepper (Jang et al., 2004; Kang et al., 1997, 2001; Lee et al., 2004; Lefebvre et al., 2001; Moon et al., 2003; Paran et al., 1998; Prince et al., 1992). These markers have proven to be very useful in assessing genetic diversity and phylogeny, characterization of germplasm and detection of duplicates, parental verification in crosses, gene tagging in marker assisted breeding and gene cloning in genetic transformation (Costa et al., 2006). The application of RFLPs for genetic diversity is limited because it requires the use of radioactivity and is labour intensive (Nahm et al., 1997). RAPDs and AFLPs identify only dominant alleles and are sensitive to PCR amplification. The working group on biochemical and molecular techniques of UPOV has identified SSR markers as the most widely used marker system for plant variety characterization (UPOV-BMT, 2002). The SSR markers are especially suitable for diversity analysis in cultivated species which have low levels of variation as detected by other types of markers and have also been used in successful prediction of heterosis and performance of $F_1$ hybrids from morphological similarity of their parents (Geleta et al., 2004). The present investigation was undertaken to characterize and give robust genetic diversity estimates in cultivated chilli peppers using SSR markers.

## MATERIALS AND METHODS

The experiment was conducted at the Vegetable Research Farm and the Vegetable Breeding Laboratory of the Department of Vegetable Science, Punjab Agricultural University, Ludhiana-141004, India during the years 2011 and 2012.

### Experimental material and the SSR markers

The experimental material comprised 64 germplasm accessions. Majority of the accessions (49) belonged to the indigenous sources and the remaining (15) to the exotic sources. Except Naga King, Tabasco and Punjab Longi, all lines belong to the species *C. annuum*. Naga King, popularly grown in Eastern India is believed to be a naturally occurring hybrid between *C. chinense* and *C. frutescens*; and Tabasco belongs to *C. frutescens*. Phylogeny of Punjab Longi is not clear but resembles that of *C. frutescens*. For diversity analysis, the germplasm was screened using 50 SSR markers of which 27 showed polymorphism (Table 1). Some of the markers used have been published (Lee et al., 2004) while others developed by AVRDC-The World Vegetable Center, Taiwan are unpublished.

### Genomic DNA extraction and SSR analysis

The genomic DNA was extracted from fresh leaf tissues following CTAB method (Saghai- Maroof et al., 1984). Quality and quantity of DNA was checked both by gel electrophoresis and spectrophotometer. *In vitro* amplification was performed in a 96 well Eppendorf Master Cycler™ (Saiki et al., 1988). The gels were visualized under UV light and photographed using photo documentation system (UV Transilluminator).

The SSR allele sizes were determined depending on the position of bands relative to the ladder (Fermantas Gene Ruler 1 KB DNA ladder). Total number of alleles was recorded for each SSR marker in all the 64 genotypes by assigning allele number as 1, 2, 3, 4 and so on. The allele amplified in a particular set having highest molecular weight was numbered as allele 1. The amplified alleles were recorded as 1 (band present) or 0 (band absent) in a binary matrix. The polymorphic information content (PIC) values for all the primers were estimated using the formula:

$$PIC = 1 - \sum_{j=1} P_{ij}^2$$

Where, $P_{ij}$ is the frequency of $j^{th}$ allele in the $i^{th}$ primer and summation extends over 'n' patterns.

### Genetic diversity and cluster analysis

The SSR marker amplification profile of 64 genotypes was used to estimate genetic similarity based on number of shared amplified

**Table 1.** Allele amplification and PIC values for the polymorphic SSR primers screened using 64 chilli pepper accessions.

| Primer | Primer sequence (5' - 3') | Number of alleles amplified | PIC Value |
|---|---|---|---|
| CAMS 117 | F ttgtggaggaaacaagcaaa<br>R cctcagcccaggagacataa | 2 | 0.44 |
| CAMS 072 | F cccgcgaaatcaaggtaat<br>R aaagctattgctactgggttcg | 3 | 0.62 |
| CAMS 885 | F aacgaaaaacaaacccaatca<br>R ttgaaattgctgaaactctgaa | 3 | 0.43 |
| CAMS 492 | F gttcaaacacttccccctca<br>R tgtcatcgttggtcgttacc | 2 | 0.49 |
| CAMS 647 | F cggattcggttgagtcgata<br>R gtgctttggttcggtctttc | 3 | 0.62 |
| CAMS 194 | F tcatggaaaattaacaacgcata<br>R gggggttggagaagaaagtt | 3 | 0.60 |
| AVRDC PP 195 | F cggtgtgctaaatagtgcca<br>R aacacaaaatgggaggtggt | 2 | 0.49 |
| AVRDC PP 166 | F gcacgaggcttcatgtca<br>R gcagcactgatcgacaaact | 3 | 0.60 |
| AVRDC PP 167 | F tcatcttacacggcttgctc<br>R agctcctcaactgcctttta | 3 | 0.65 |
| AVRDC PP 154 | F cttcctagccacacacctca<br>R gagcccaaaattcaaccagt | 3 | 0.65 |
| AVRDC PP 208 | F cccctatctctttgctgctt<br>R agctggggttttacaaatgg | 3 | 0.63 |
| AVRDC PP 65 | F gtgaggccgagaatgaagat<br>R aacgaccatgtgtggttga | 3 | 0.65 |
| AVRDC PP 67 | F tattccttcttcacccctcc<br>R gaaagaggcgctaactggac | 3 | 0.58 |
| AVRDC PP 3 | F ctcgatgacttgatcgtga<br>R cttgcattgtgaggtcactg | 3 | 0.66 |
| AVRDC PP 17 | F ctactaccgctcctgctcct<br>R agcttctgcttttggttcgt | 3 | 0.64 |
| AVRDC PP 18 | F gctaggcttgatccttcacc<br>R cgcttgaaatcatgctcact | 3 | 0.78 |
| AVRDC PP 24 | F aaagcatgaaatcaccctcc<br>R cggcaagaagatgaaagtca | 3 | 0.66 |
| AVRDC PP 32 | F atggaggattacctcgcaac<br>R catgatgaccatccatccat | 4 | 0.69 |
| AVRDC PP 205 | F aacccccttcaaacttgttgc<br>R gggggttcgaagtagatgaa | 3 | 0.63 |
| AVRDC PP 157 | F gaattagctgcaacccaaca<br>R gatttgtgatgccaccagac | 3 | 0.64 |
| AVRDC PP 174 | F tcgttgttgggtggtacttg<br>R ggaagatctcaaatgggtcg | 2 | 0.50 |

**Table 1.** Contd.

| | | | |
|---|---|---|---|
| AVRDC PP 187 | F atcgtcgtcatccccatatt<br>R aagataccatgccccttctg | 3 | 0.39 |
| AVRDC PP 188 | F ctgctcttgaaaccgttgaa<br>R cctctccatgaccctcctta | 2 | 0.50 |
| AVRDC PP 227 | F attgattctgttggtggggt<br>R ttccgtgtatcgctgctaac | 2 | 0.48 |
| AVRDC PP 239 | F caaatgctgccactcacttt<br>R acaacaaggggtgtttcctc | 3 | 0.66 |
| CAMS 679 | F tttgcatgttttacccattcc<br>R ccccaaaaattttccctcat | 3 | 0.66 |
| CAMS 806 | F ggaccgttcaggaggttaca<br>R gccatcattcaaaaccgaat | 3 | 0.61 |
| Total<br>Average | | 75<br>2.78 | -<br>0.59 |

Monomorphic primers were not included for estimating total and average value.

bands. The presence or absence of a particular amplification product was used as an index of genetic diversity/ relatedness using computer software package Windostat version 8.6. Clustering was done by UPGMA using SHAN module of Windostat version 8.6.

## RESULTS AND DISCUSSION

Out of 50 primers screened, 23 primers did not show polymorphism and were not considered for further analysis. Twenty seven (27) primers were thus used for genetic diversity analysis on the basis of scoreable amplified bands. The number of bands amplified by each of the 27 primers ranged from two to four on superfine 2.5% agarose gel. A total of 75 alleles with an average of 2.78 alleles per locus were amplified in 64 genotypes. Maximum of four alleles were observed for primer AVRDC PP 32 and majority of the primers (20) amplified three alleles each (Table 1). The remaining six primers amplified two alleles each.

The PIC values provide an estimate of discriminating power of a marker by taking into account not only the number of alleles at a locus but also relative frequencies of these alleles. These values depend upon the genetic diversity among the accessions. Lower PIC values might be the result of closely related genotypes and the *vice-versa*. Senior et al. (1998) opined that marker loci with an average number of alleles running at equal frequencies will have the highest PIC value. The PIC values obtained in the present study varied from 0.39 for AVRDC PP 187 to 0.78 for AVRDC PP 18, with an average PIC value for 27 polymorphic primers to be 0.59. Based on the PIC values, it was found that primers AVRDC PP 18 was the most informative (0.78), followed by primers AVRDC PP 32 (0.69) and AVRDC PP 03 (0.66) whereas AVRDC PP 187, CAMS 117 and CAMS 885 with PIC values 0.39, 0.44 and 0.43, respectively, were the least informative. Our results were similar to those of Kwon et al. (2005) (0.53) and Yumnam et al. (2012) (0.52).

The SSR analysis revealed that the polymorphic level in this research was fairly high (54%) compared to some earlier reports indicating higher levels of genetic diversity among Indian accessions. For example, Paran et al. (1998) using 10 primer pairs detected 13% polymorphism in 34 Israeli gene bank accessions; Akatas et al. (2009) using four primer pairs found 26% polymorphism in Turkish germplasm; Kochieva and Ryzhova (2003) using nine primer pairs found 16.5% polymorphism in 14 Russian breeding lines; and Tam et al. (2005) using nine primer pairs observed 8.03% polymorphism. However, other workers using RAPD and AFLP techniques reported comparatively higher level of polymorphism. Oyama et al. (2006) screened the wild and domesticated pepper (*C. annuum* L.) populations of North-western Mexico where wild populations of *C. annuum* L. are widely distributed. Using RAPD markers, they reported higher levels of polymorphism in wild (all 166 band polymorphic) and domesticated (125 of 126 band polymorphic) populations. Geleta et al. (2005) obtained 352 polymorphic markers in the analysis of 39 accessions using six AFLP primer pairs. Using only four AFLP markers, Aktas et al. (2009) observed 215 bands, fifty-six (26%) of them were polymorphic indicating that AFLP markers are more efficient than the other marker systems. The varying levels of polymorphism in chilli pepper reported by various research groups could be attributed to the differences in genetic structures of the populations screened and the molecular techniques used. Samples collected from natural habitats of Mexico were expected to reveal higher levels of variability.

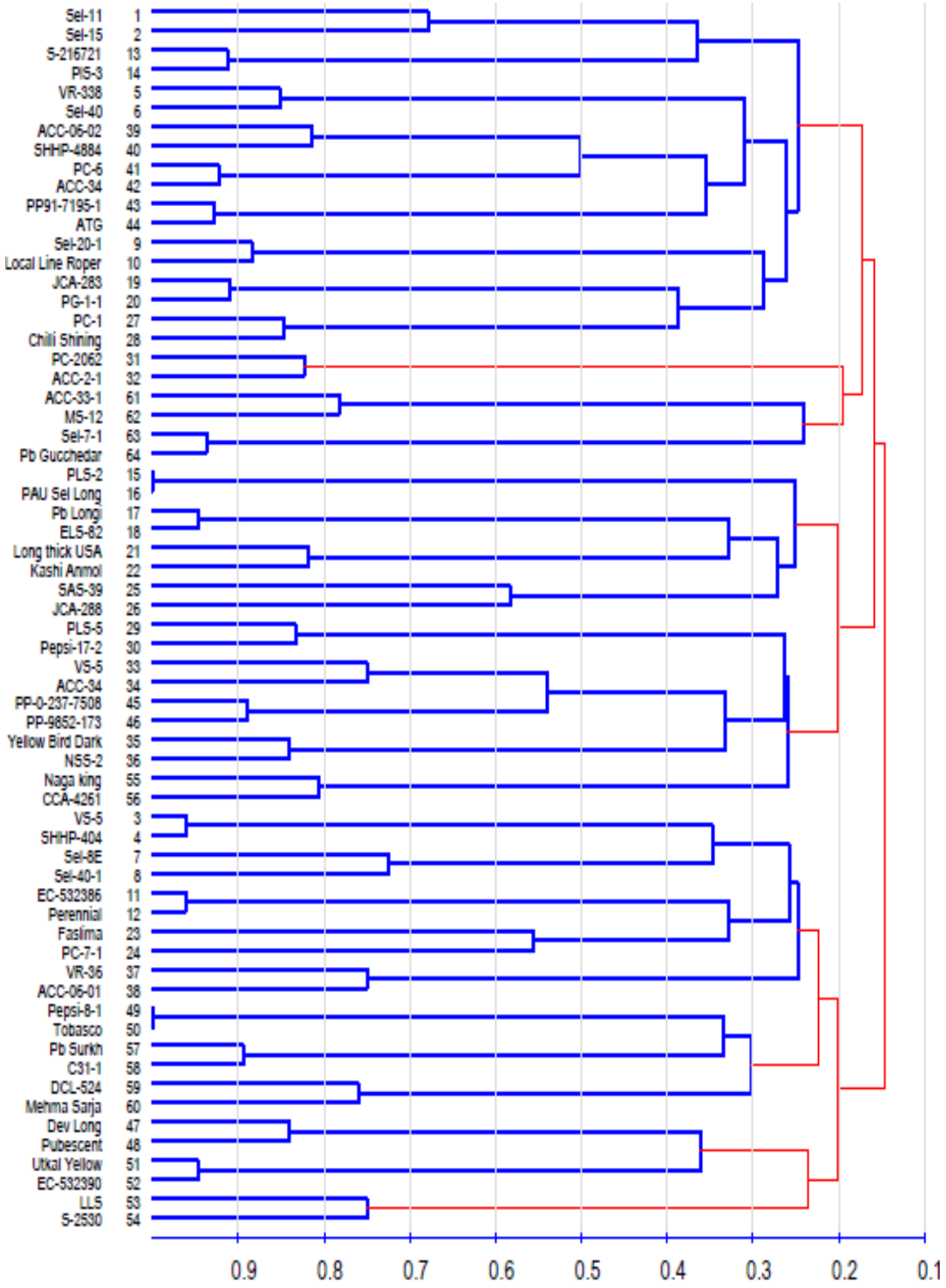


**Figure 1.** UPGMA based dendrogram of 64 chilli pepper genotypes based on 27 polymorphic SSR markers.

The diversity coefficient between any two genotypes estimated based on DNA amplification by SSR primers varied from 0.00 (between Pepsi 8-1 and Tabasco; and PLS-2 and PAU Selection Long) to 1.00 (between PC 2062 and Punjab Longi). The dendrogram showing genetic relationships among 64 genotypes based on SSR markers is presented in Figure 1. The UPGMA cluster analysis showed that all the 64 pepper genotypes were

clustered in to two main groups at similarity coefficient of 0.85. The larger group comprised of 42 genotypes and the smaller group comprised of 22 genotypes. Finally, the 64 genotypes were divided into nine sub- groups at similarity co-efficient of 0.75. The largest sub-group (Cluster 1) comprised of 18 genotypes and the smallest sub-groups (clusters 2 and 9) comprised of two genotypes each. The molecular analysis revealed that 222 pairs of genotypes have diversity coefficient of 0.90 or more, whereas only 12 pairs of genotypes have diversity coefficient of 0.10 or less.

Based on the molecular analysis, it was possible to characterize most of the accessions studied. However, the molecular analysis could not differentiate PLS 2 from PAU Sel Long; and Pepsi 8-1 from Tabasco. The probable reasons could be either due to the duplicates in the germplasm under different names or the present set of markers is not sufficient to detect differences between the two pairs of genotypes or due to the technological limitations. Though, the qualitative characters are subjective and hard to score; and the quantitative characters are influenced by the environment, yet it is unlikely that the two pairs of genotypes with contrasting morphological features are duplicates. Tabasco bears small erect fruits where as Pepsi 8-1 has medium long pendent fruits. Similarly, PLS 2 have broad leaves; and comparatively larger and pungent fruits, whereas PAU Sel Long have small leaves; and medium sized and mildly pungent fruits (Yadav, 2013). Molecular analysis with additional SSR markers and with greater genome coverage could help to reveal genetic diversity accurately and help to unambiguously differentiate those accessions with identical allelic patterns as revealed by the current set of SSR markers. The markers developed specifically for *C. annuum* and *C. frutescens* might give better results to differentiate Pepsi 8-1 from Tabasco, as the SSR markers some times are species specific. Another possible approach could be to go for AFLP markers which can reveal high frequency of DNA polymorphism even within cultivars as the genome coverage of these markers can be very high compare to SSR markers (Paran et al., 1998; Aktas et al., 2009; Kochieva and Ryzhova, 2003; Tam et al., 2005).

In the past, SSR markers have been used in successful prediction of heterosis and performance of $F_1$ hybrids from morphological similarity of their parents (Geleta et al., 2004). Due to facultative breeding nature (Tanksley, 1984) and availability of male sterility, it is easier to develop hybrids in chilli pepper. The current list of germplasm included accessions possessing both nuclear (NMS) and cytoplasmic (CMS) male sterility systems. Following this technique, parents have been identified for heterosis breeding. The pollen parents namely Pepsi 17-2, Acc 34, Utkal Yellow, EC 532390, Punjab Surkh, C 31-1, NSS 2 and VR 36 were found to be divergent from the CMS line PP-91-7195, with diversity coefficient of 0.90 or more. Similarly, Sel 7-1 and Punjab Guchhedar were found to be divergent from the CMS line PP 9852- 17; Punjab Guchhedar from PP 0-237-7508; Sel 11, LLS and DCL 524 from CCA 4261; PLS 2 and PAU Sel Long from PP-0-237-7; and ACC 06-01, Dev Long, Pubescent, Pepsi 8-1 and Tabasco from the NMS line MS 12. Due to their genetic divergence, the identified parents are expected to produce heterotic hybrids. Based on diversity analysis, parents Punjab Longi and PC-2062; Acc-0601 and Long Thick USA; Acc-0601 and Kashi Anmol; Yellow Bird Dark and LLS, PP 91-7195-1 and C 31-1; and ATG and C 31-1 with diversity coefficient of 0.97 or above were identified to attempt crosses and develop populations for mapping of useful genes, estimation of gene effects and to breed superior performing crop cultivars.

## Conclusion

Our results based on the SSR analyses have vindicated that India is an important source of genetic variability of cultivated peppers, especially of hot pepper. The current set of primers is adequate to differentiate and characterize most of the chilli pepper genotypes studied. Based on the diversity analysis, parents have been identified for developing mapping populations, crop improvement and heterosis breeding.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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*Review*

# Genetic variation and DNA markers in forensic analysis

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**In this review, we highlighted the DNA markers and their applications in forensic medicine which included the study of genetic variation or polymorphism, markers for genetic variation, types of variation, diallelic polymorphisms (SNPs and Indels), haplotypes, autosomal short tandem repeat (STRs), Y-chromosome short tandem repeat (Y-STRs) and Y chromosome. This is a useful tool for tracing human evolution, analysis of Y-chromosomal microsatellite haplotypes in globally human populations, organization of the human mitochondrial genome, and mitochondrial coding region as a source for variability and interpretation of sequence data. The light has been focused and directed in this study to establish the basic forensic genetic information, knowledge, data and statistics which might be so ultimately helpful practically in forensic science and criminology and to let evaluate and present the DNA weight evidences in medico-legal institute and courts of law.**

**Key word:** Applications, DNA markers, forensic, genetic variation, review.

## INTRODUCTION

Every organism stores its information on identity in its DNA (Dobbs et al., 1994). Separate chromosomes which are available in the nucleus of cells of humans have DNA. Serological and DNA typing are being used in forensic biology to analyze evidence found at crime scenes. The sources biological evidences may be bodily fluids or tissues (Pierce and Wangh, 2007).

Microsatellites are a group of molecular markers chosen for a number of purposes which include forensic individual identification and relatedness testing

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**Abbreviations: AMH,** Anatomically modern human**; bp/kbp,** base pair/thousand (kilo) base pairs; **CRS,** Cambridge reference sequence; **D-loop,** displacement loop/control region of mtDNA; **DNA,** deoxyribonucleic acid; hg(s), haplogroup(s); **HVS-I/HVS-II,** the first/second hypervariable segment; **LD,** linkage disequilibrium; **LGM,** last glacial maximum; **MRCA,** most recent common ancestor; **mtDNA,** mitochondrial DNA; **np(s),** nucleotide position(s); **NRY,** non-recombinant Y chromosomes; **OXPHOS,** oxidative phosphorylation; **PCR,** polymerase chain reaction; **RFLP,** restriction fragment length polymorphism; **GD,** gene diversity; **HWE,** Hardy-Weinberg equilibrium; **ISFG,** International Society of Forensic Genetics; **PD,** power of discrimination; **PE,** power of exclusion; **PI,** paternity index; **PIC,** polymorphism informative content; **PM,** match probability; **Pr,** probability; **SNP,** single nucleotide polymorphism; **STR,** short tandem repeat.

(Yamamoto et al., 1999; Nakamura et al., 2009). Low quantities of template DNA are required (10-100 ng) (Markoulatos et al., 2002), when using microsatellites. There is a high genomic abundance of random distribution throughout the genome. There is also an abundance of polymorphism. A nuclear DNA present in one copy per cell and only in males is called the Y chromosome. It includes the sex determining region and known as a paternal lineage marker (Butler et al., 2002; Carolina et al., 2010; Kuppareddi et al., 2010). The genetic information is inherited from the father to the son, and this information does not change except for mutational events (Hanson and Ballantyne, 2007; Mohammad and Imad, 2013). The individual short tandem repeat (STRs) are inherited as a single unit because of the lack of recombination which is called a haplotype and behaves as single allele per individual (Parson et al., 2003; Kwak et al., 2005).

The Y - chromosome is specific to the male portion of a male-female DNA mixed such as is common in sexual assault cases (Park et al., 2007). These STRs can also be useful in missing persons investigations, historical investigations, some paternity testing scenarios, and genetic genealogy (Park et al., 2007; Andrea et al., 2008). Although they are often used to suggest which haplogroup an individual matches, STR analysis typically provides a person haplotype. Most tests on the Y chromosome examine between 12 and 67 STR markers (Kayser et al., 1997; Imad et al., 2013a). The Y chromosome is less variable than the other chromosomes. Many markers are thus needed to obtain a high degree of discrimination between unrelated males (Kuppareddi et al., 2010; Hanson and Ballantyne, 2007).

The mitochondrial DNA (mtDNA) is a small circular genome located within the mitochondria in the cytoplasm of the cell. The mitochondrial genome can be divided into two sections: a large coding region, which is responsible for the production of various biological molecules involved in the process of energy production in the cell, and a smaller 1.2 kb pair fragment, called the control region. It is found to be highly polymorphic and harbors three hypervariable regions (HV): HV1, HV2 and HV3 (Kraytsberg et al., 2004; Imad et al., 2013b). Mitochondrial DNA Comprising of about 37 genes coding for 22 tRNAs, two rRNAs and 13 mRNAs are a small circle of DNA (Helgason et al., 2003).

Mitochondrial DNA does not recombine and thus there is no change between parent and child, unlike nuclear DNA. MtDNA is only passed on from mother to child and this is an important fact (Ingman et al., 2003; Ukhee et al., 2005). There is more sequence divergence in mitochondrial than in nuclear DNA (Brown et al., 1993; Giulietta et al., 2000). This may be caused by a faster mutation rate in mtDNA that may result from a lack of repair mechanisms. Sequencing of highly polymorphic segments of the control region of mitochondrial DNA (mtDNA) is today a routine method of analysis of biological traces which are not suitable for STR analysis due to insufficient concentration of nuclear DNA or heavy degradation processes (Holland, Parsons, 1999; Tzen et al., 2001). A promising approach in this context seems to be analysis of selected single nucleotide polymorphisms (SNPs) that are useful for identification purposes.

## GENETIC VARIATION OR POLYMORPHISM

The following: effective population size, population history (migration, bottleneck, and recent expansion), population structure and location of diseases genes are determined by the amount and nature of genetic variation in a population. There are three items: (1) changes in nucleotides which could be transition or transversion. In the transition mutation, a pyrimidine (C or T) is substituted by another pyrimidine, or a purine (A or G) is substituted by another purine. The transversion mutation involves the change from a pyrimidine to a purine, or vice versa; (2) insertion or deletion of single nucleotides (indel) and (3) variation in number of repeat of tandemly repeated sequences (microsatellite, minisatellite and satellite) are genetic variation or polymorphism (Roach, 2010; Conrad, 2011).

The study of human genetic variation has both evolutionary significance and medical applications. It can help scientists understand ancient human population migrations as well as how different human groups are biologically related to one another. For medicine, study of human genetic variation may be important because some disease-causing alleles occur more often in people from specific geographic regions (Tishkoff and Verrelli, 2003; Conrad, 2011).

### Diallelic polymorphisms (SNPs and INDELS)

Single-nucleotide polymorphisms (SNPs), the occurrence of different nucleotides at a specific place in the genome, are the most common type of human DNA sequence variation, occurring on average 1 per 500 to1000 bp on a randomly selected chromosome. As noted later, this number varies somewhat depending on the population studied. Insertion/deletion polymorphisms (Indels), the occurrence of some more or fewer nucleotides at one position of the genome, are less common but do occur frequently.

The inserted/deleted sequence can be a few nucleotides long (Weber et al., 2002) up to several hundred nucleotides long, as is the case with the transposable Alu elements (Carter et al., 2004). Some very large polymorphic duplications, hundreds of kilobase pairs long, have also been identified (Sebat et al., 2004; Iafrate et al., 2004). Because these polymorphisms are diallelic, they are less informative than microsatellites; however, SNPs are more common in the genome and more

amenable to automation and DNA chip technology. Most of the RFLPs (restriction fragment length polymorphisms) defined between the late 1970s and the late 1990s were SNPs in a restriction site but some Indels were occurring between restriction sites. That is, they are caused by a single mutational event that occurred once in the history of a species. We do not expect to see recurrent mutations at the same site except as extremely rare events. For the polymorphic Alu elements, we know that the ancestral state is absence and the insertion of the Alu is the derived state. For the other markers, we cannot tell from the human polymorphism which allele is ancestral and which is derived. However, we can determine the ancestral state in almost all of those cases simply by determining the genotype of our nearest relatives, the other great apes, following the logic in most cases. Humans share a single allele with the other apes and, by inference this is the ancestral or original allele in humans and the other allele is the derived allele (Hacia et al., 1999).

### Multiallelic polymorphisms (STRPs and VNTRs)

Microsatellites consist of approximately 10-50 tandemly repeated copies of particular DNA sequence motifs ranging from 1 to 10 (most commonly 2-4) nucleotide base pairs. These repeat sequences, discovered in 1989 (Litt and Luty, 1989), occur frequently and randomly across the human genome. When the repeat number is polymorphic, microsatellites are also called short tandem repeat polymorphisms (STRPs) (other acronyms have also been used, for example, SSLP). STRP loci usually have multiple alleles and can have high levels of variation, that is, high heterozygosity. They rapidly replaced RFLP markers in gene mapping (especially disease genes) studies owing to these features and to their ease of typing, including the small amount of template DNA required. Remember that heterozygosity can never exceed 50% for a diallelic marker, whereas STRPs can easily have heterozygosities >75%. Because of this high heterozygosity, STRPs are the markers on which the most detailed human linkage maps are based (Dib et al., 1996; Kong et al., 2002; Jorgenson et al., 2005). STRPs have also become the standard for forensics and paternity testing (Budowle et al., 2001). The larger minisatellite arrays (also referred to as VNTRs, variable number of tandem repeats) are also highly polymorphic and powerful markers in forensic and paternity studies (DNA fingerprinting) (Jeffreys et al., 1985; Armour et al., 1996). However, they are less common than STRPs and are not evenly distributed throughout the genome. Their larger sequence motifs make them less amenable to PCR technology and use in genomic screening analyses. STRPs and VNTRs, on the other hand, tend to be much more dynamic than the diallelic SNPs and Indels. The mutation rates are higher, there are more alleles, and it is usually impossible to determine which is the ancestral allele.

### AUTOSOMAL SHORT TANDEM REPEAT (STRs)

Microsatellites refer to DNA with varying numbers of short tandem repeats (Allor et al., 2005; Klintschar et al., 2006) between a unique sequence (Figures 1 and 2). DNA regions with repeat units that are 2 to 7 bp in length or most generally short tandem repeats (STRs) or simple sequence repeats (SSRs) are generally known as microsatellites (Ellegren, 2004). In the core repeated bases, long repeat units may contain several hundred to thousands (Butler and Hill, 2012). Within the DNA there are length and sequence polymorphisms (Silvia et al., 2009). DNA can be used to study human evolution using human genome analysis regions that are not subjected to selection pressure (Mats et al., 2007). Besides, information from DNA typing provides vital information in medico-legal with polymorphisms allowing for more biological studies (Walkinshaw et al., 1996). It has been found that microsatellites are evenly distributed in the genome on all chromosomes and all regions of the chromosome (Ensenberger et al., 2010). They can also be found inside gene coding regions, introns, and in the non-gene sequences. Most microsatellite loci are really small, ranging from a few to a few hundred repeats and this small size of microsatellite loci is important for PCR-facilitated genotyping. Basically microsatellites containing a higher number of repeats are more polymorphic.

### STR LOCUS NOMENCLATURE

The nomenclature for DNA markers is fairly straightforward. If a marker is part of a gene or falls within a gene, the gene name is used in the designation. For example, the STR marker TH01 is from the human tyrosine hydroxylase gene located on chromosome 11. The ‘01’ portion of TH01 comes from the fact that the repeat region in question is located within intron 1 of the tyrosine hydroxylase gene. Sometimes the prefix HUM- is included at the beginning of a locus name to indicate that it is from the human genome. Thus, the STR locus TH01 would be correctly listed as HUMTH01 (Butler, 2005). DNA markers that fall outside of gene regions may be designated by their chromosomal position. The STR loci D5S818 and DYS19 are examples of markers that are not found within gene regions. In these cases, the ‘D’ stands for DNA. The next character refers to the chromosome number, 5 for chromosome 5 and Y for the Y chromosome. The ‘S’ refers to the fact that the DNA marker is a single copy sequence. The final number indicates the order in which the marker was discovered and categorized for a particular chromosome. Sequential numbers are used to give uniqueness to each identified

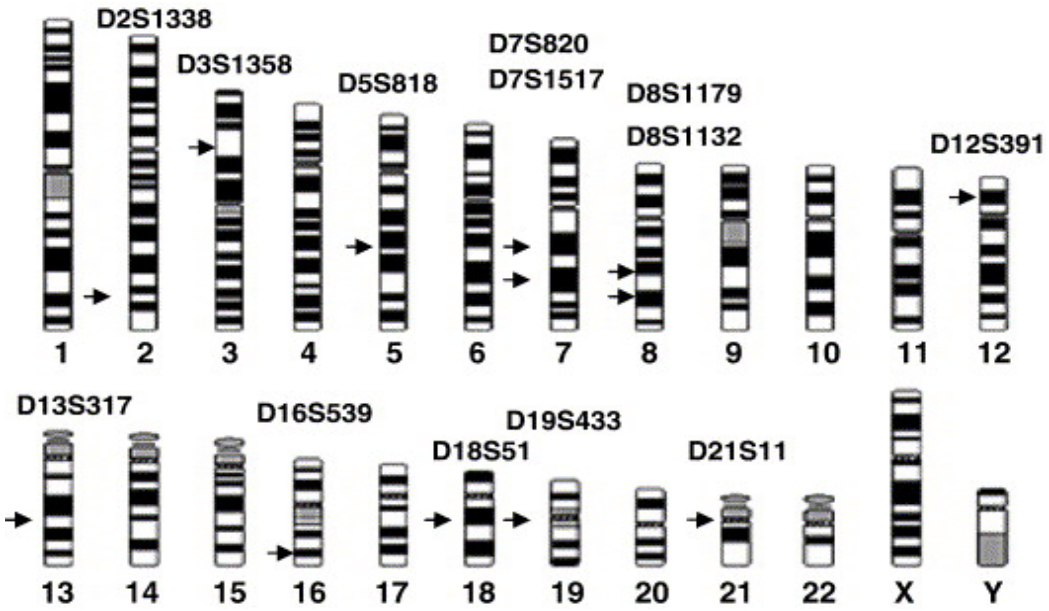


**Figure 1.** Exact physical location of 13 STR markers called CODIS : D2S1338 (q35), D3S1358 (3p21.31), D5S818 (5q23.2), D7S820 (7q21.11), D7S1517 (7q31.33), D8S1179 (8q24.13), D8S1132 (8q23.1), D12S391 (12p13.2), D13S317 (13q31.1), D16S539 (16q24.1), D18S51 (18q21.33), D19S433 (19q12), D21S11 (21q21.1). (Butler, 2006).

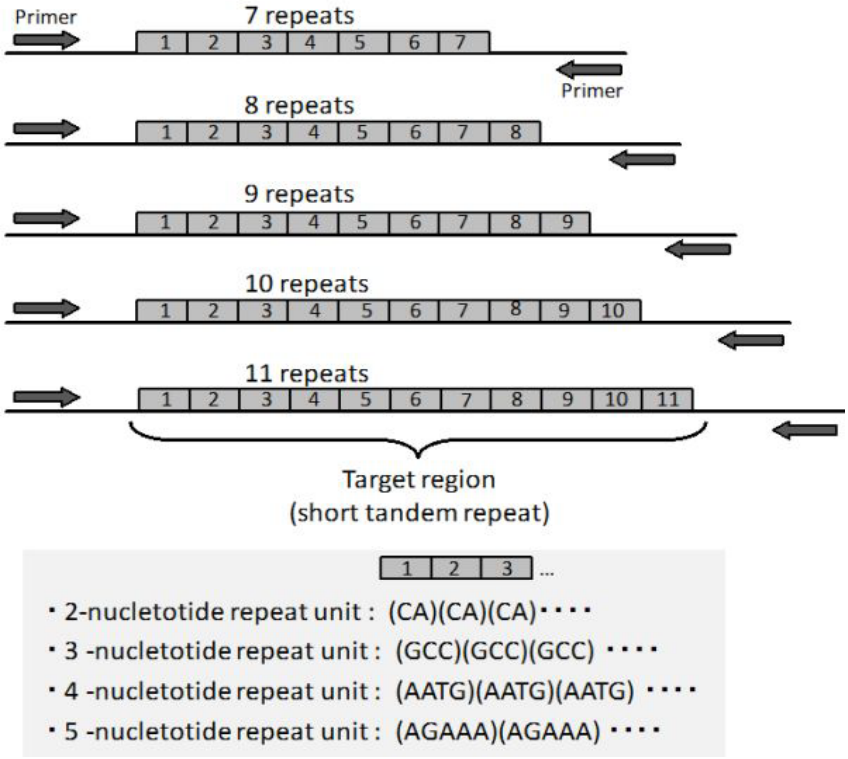


**Figure 2.** The structure of Short Tandem Repeat (STR) (Butler, 2006).

DNA marker (Butler, 2005). Thus, for the DNA marker D3S1358: D = DNA, 3 = Chromosome 3, S = single copy sequence, 1358 = 1358th locus described on chromosome 3.

**Table 1.** Comparison of STR loci present in kits used in the United States.

| Fix parameter here | Power Plex®18D | Power Plex®21 | Power Plex®Fusion | Identifiler | Mini Filer | Extended[a] |
|---|---|---|---|---|---|---|
| TPOX | TPOX | TPOX | TPOX | TPOX | - | - |
| CSF1PO | CSF1PO | CSF1PO | CSF1PO | CSF1PO | CSF1PO | CSF1PO |
| D5S818 | D5S818 | D5S818 | D5S818 | D5S818 | - | D5S818 |
| D7S820 | D7S820 | D7S820 | D7S820 | D7S820 | D7S820 | D7S820 |
| D13S317 | D13S317 | D13S317 | D13S317 | D13S317 | D13S317 | D13S317 |
| FGA | FGA | FGA | FGA | FGA | FGA | FGA |
| vWA | vWA | vWA | vWA | vWA | - | vWA |
| D3S1358 | D3S1358 | D3S1358 | D3S1358 | D3S1358 | - | D3S1358 |
| D8S1179 | D8S1179 | D8S1179 | D8S1179 | D8S1179 | - | D8S1179 |
| D18S51 | D18S51 | D18S51 | D18S51 | D18S51 | D18S51 | D18S51 |
| D21S11 | D21S11 | D21S11 | D21S11 | D21S11 | D21S11 | D21S11 |
| TH01 | TH01 | TH01 | TH01 | TH01 | - | TH01 |
| D16S539 | D16S539 | D16S539 | D16S539 | D16S539 | D16S539 | D16S539 |
| - | D2S1338 | D2S1338 | D2S1338 | D2S1338 | D2S1338 | D2S1338 |
| - | D19S433 | D19S433 | D19S433 | D19S433 | - | D19S433 |
| - | - | D12S391 | D12S391 | - | - | D12S391 |
| - | - | D1S1656 | D1S1656 | - | - | D1S1656 |
| - | D2S441 | - | - | - | - | D2S441 |
| - | - | - | D10S1248 | - | - | D10S1248 |
| - | - | D6S1043 | - | - | - | - |
| - | - | - | D22S1045 | - | - | - |
| Penta D | Penta D | Penta D | Penta D | - | - | - |
| Penta E | Penta E | Penta E | Penta E | - | - | Penta E |
| - | - | - | DYS391 | - | - | DYS391 |
| Amelogenin | Amelogenin | Amelogenin | Amelogenin | Amelogenin | Amelogenin | Amelogenin |

[a](*see* Ref. (Lareu et al., 1996) for more information on the proposed U.S. extended core).

## COMMON COMMERCIAL KITS USED IN THE UNITED STATE

To further assist efforts in forensic DNA testing and human identification, Promega and Applied Biosystems have released a number of new STR typing kits Table 1. The European Standard Set of STR loci added to U.S. core loci used for the Combined DNA Index System are among the most recent STR kits were developed. Some of these kits are capable of simultaneous, multicolor fluorescence detection of 15 STRs and the sex-typing marker amelogenin in a single PCR reaction (Butler et al., 2006).

## AUTOSOMAL STRs LOCUS INFORMATION

Autosomal STRs locus information were evaluated and selected at The Cooperative Human Linkage Center. http://www.chlc.org evaluates the genetic markers and the loci are selected from there. Additional STR loci, chromosomal location and repeat sequence for each core STR locus are provided in Table 1 and Table 2 (Ruitberg et al., 2001; Klintschar et al., 2005; Klintschar et al., 2006; Imad et al., 2014a). According to the International Society of Forensic Genetics (ISFG) recommendation the repeat sequence motif be defined so that the first 5'-nucleotides on the Gene Bank forward strand define the repeat motif used. So the repeat motif for each STR marker is listed based on this. A significant fact is that STR allele sizes are measured relative to an internal size standard during electrophoresis. This depends on the DNA strand that is labeled using a dye that may have a different apparent measured size.

## Y-CHROMOSOME SHORT TANDEM REPEAT (Y-STRs)

Chromosome Y microsatellites or STR's seem to be an ideal markers to delineate differences between human populations for several reasons: (i) they are transmitted in uniparental (paternal) fashion without recombination (De Knijff et al., 1997), (ii) they are very sensitive for genetic

**Table 2.** Information on 21 autosomal STR loci present in The PowerPlex® 21 System kits

| STR Locus[a] [n] | Label | Physical position | Chromosomal location1 | Repeat sequence 5´-3´ |
|---|---|---|---|---|
| Amelogenin | Fluorescein | X and Y | Xp22.1-22.3 and Y | NA |
| D3S1358 | Fluorescein | Chr 3 (45.582 Mb) | 3p21.31 | TCTA Complex |
| **D1S1656** | **Fluorescein** | Chr 1 (230.905 Mb) | 1q42 | TAGA Complex |
| **D6S1043** | **Fluorescein** | **Chr 6 (92.450 Mb)** | **6q15** | **AGAT** |
| D13S317 | Fluorescein | **Chr 13 (82.692 Mb)** | **13q31.1** | **TATC** |
| **Penta E** | **Fluorescein** | Chr 15 (97.374 Mb) | 15q26.2 | AAAGA |
| D16S539 | JOE | **Chr.16(86.386Mb)** | **16q24.1** | **GATA** |
| D18S51 | JOE | Chr 18 (60.949 Mb) | 18q21.33 | AGAA |
| D2S1338 | JOE | Chr 2 (218.879 Mb) | 2q35 | TGCC/TTCC |
| CSF1PO | JOE | Chr 5 (149.455 Mb) | 5q33.1 | AGAT |
| **Penta D** | **JOE** | **Chr 21 (45.056 Mb)** | **21q22.3** | **AAAGA** |
| TH01 | TMR-ET | Chr 11 (2.192 Mb) | 11p15.5 | AATG (19) |
| vWA | TMR-ET | Chr12(6.093 Mb) | 12p13.31 | TCTA Complex (19) |
| D21S11 | TMR-ET | Chr 21 (20.554 Mb) | 21q21.1 | TCTA Complex (19) |
| D7S820 | TMR-ET | Chr 7 (83.789 Mb) | 7q21.11 | GATA |
| D5S818 | TMR-ET | Chr 5 (123.111 Mb) | 5q23.2 | AGAT |
| TPOX | CXR-ET | Chr 2 (1.493 Mb) | 2p25.3 | AATG |
| D8S1179 | CXR-ET | Chr 8 (125.907 Mb) | 8q24.13 (125.976 Mb) | TCTA Complex (19) |
| **D12S391** | **CXR-ET** | Chr 12 (12.450 Mb) | **12p12(12.341 Mb)** | AGAT/AGAC **Complex** |
| D19S433 | CXR-ET | **Chr19(30.416 Mb)** | (35.109 Mb) | AAGG Complex |
| FGA | CXR-ET | Chr 4 (155.509 Mb) | 4q28 (155.866 Mb) | 4q28 (155.866 Mb) |

[a]database of sequence-tagged sites (STSs) available on the NCBI website: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unists. [n]The 13 CODIS core loci are highlighted in bold font. Adapted from Cotton et al., 2000; Wiegand et al., 1993; physical positions are from Schneider et al., 1998.

drift, and (iii) they allow a simple highly informative haplotype construction (Kayser et al., 1997). Also, for forensic applications, this ability to differentiate distinct Y chromosomes makes Y-STR's an advantageous addition to the well characterized autosomal STR's. For a number of forensic applications Y-STR's could be superior to autosomal STR's. Especially in rape cases where (i) the differential extraction was unsuccessful, (ii) the number of sperm cells is very low, (iii) due to vasectomy epithelial cells instead of sperm cells from the ejaculate of the perpetrator have to be analyzed, and (iv) the perpetrator, due to a familial relationship shares many autosomal bands with the victim, Y-STR's could provide crucial evidence. Also, in the case of male-male rape or rape cases with multiple perpetrators Y-STR's could lead to essential qualitative evidence. In all such cases Y-STR's facilitates a simple and reliable exclusion of suspects (Redd et al., 1997).

## COMMON COMMERCIAL KITS USED IN THE UNITED STATES

Two most popular commercial kits in the U.S., are namely PowerPlex®Y System (a,b) and AmpFlSTR®Yfiler™ PCR Amplification Kit. There are currently four online searchable Y-STR haplotype databases based in the United States and intended for forensic use. Three are maintained by commercial vendors: Reliagene, Inc., Promega Corporation and Applied Biosystems, Inc. The fourth is maintained by the University of Arizona. The National Center for Forensic Science (NCFS) also maintains a Y-STR database that will soon be available online. However, these databases are somewhat limited in the number of individuals and loci profiled, which sometimes limits their operational usefulness.

## Y CHROMOSOME IS A USEFUL TOOL FOR TRACING HUMAN EVOLUTION

The Y chromosome is becoming a useful tool for tracing human evolution through male lineages (Jobling, 1994) as well as application in a variety of forensic situations (Kayser et al., 1997) including those involving evidence from sexual assault cases containing a mixture of male and female DNA (Prinz et al., 1997; Prinz et al., 2001). Y-chromosome specific methods can improve the chances of detecting low levels of male DNA in a high background of female DNA.

The European Y chromosome typing community has

established a “minimal haplotype” and an “extended haplotype” for inclusion of common loci into a central DNA database. These haplotypes consist of results from the following Y STR markers: DYS19, DYS389I, DYS389II, DYS390, DYS391, DYS392, DYS393, DYS385, and YCAII (Roewer et al., 2001). The combination of these Y STRs can distinguish approximately 74 to 90% of male individuals in various local European populations (Kayser et al., 1997). The minimal haplotype, the results of which have been accepted for court use in Europe (Pascali et al., 1999), is most commonly used and is typically obtained via two or three separate multiplex amplifications. In this study, a Y STR 20 plex capable of the simultaneous amplification of 20 polymorphic Y chromosome-specific PCR products is introduced. This 20 plex includes all of the markers in the European extended haplotype (Roewer et al., 2001) and also contains the trinucleotide loci DYS388 (M. Kayser et al., 1997) and DYS426 (Jobling et al., 1996), the tetranucleotide loci DYS437 (Ayub et al., 200; Grignani et al., 2000), DYS439 (Ayub et al., 2000; Grignani et al., 2000), GATA A7.1 (DYS460) and H4 (White et al.,1999; Gonzalez-Neira et al., 2001), the pentanucleotide loci DYS438 (Ayub et al., 2000; Grignani et al., 2000) and DYS447 (Redd et al., 2002), and the hexanucleotide marker DYS448.

## Analysis of Y-chromosome microsatellite haplotypes in globally human population

Variability at microsatellite or short tandem repeat (STR) loci is being used, in various species, for linkage analysis, individual identification (Hammond et al., 1994; Redd et al., 1997), and population-genetic analyses. Autosomal STR loci have also been successfully applied to reconstruct human evolutionary history (Goldstein et al., 1995). The resulting phylogenetic trees reveal evolutionary relationships similar to those based on mtDNA sequence variation, and it has been a long lasting wish to add to these trees the one strictly based on Y chromosome- specific markers. Approximately 5 years ago, the number of published STR loci on the human Y chromosome was <15, and only oneY chromosome-specific minisatellite was known (Roewer et al., 1992). The number of known Y chromosomal single-nucleotide polymorphisms (SNPs) was <10 (Nakahori et al., 1989; Jobling, 1994; Mathias et al., 1994; Seielstad et al., 1994; Hammer, 1995; Whitfield et al., 1995), and only one Alu-insertion polymorphisms had been discovered. This picture, however, has totally changed, owing to the recent introduction of many Y SNPs (Underhill et al., 2000) and STRs (White et al., 1999), and additional new markers are to be expected.

Recently, Y-STR variability has been used both for the dating of SNP mutations, in order to draw conclusions about the origins and history of human populations (Underhill et al., 1996; Zerjal et al., 1997), and for human identification in forensic casework (Kayser et al., 1997; Prinz et al., 1997). Nevertheless, global studies of Y-STR variability are still rare (Seielstad et al., 1999), and most of these analyses have been based on combined single-locus information. The major advantage of analyzing the nonrecombining part of the Y chromosome is that single-locus information can be used to construct compound haplotypes that allow male lineages to be characterized in a much more detailed fashion. It has been questioned whether the tracing of human migration history can be achieved solely on the basis of Y STRs (De Knijff et al., 1997).

## Y-STR database

When the DNA profile of a known suspect or victim matches the DNA profile from crime scene evidence, the individual is "included" as a potential source of that evidence. In the U.S., the strength of the match is most often expressed as a statistic that describes the estimated frequency of occurrence of the DNA profile in unrelated individuals within various population groups. Due to the lack of recombination along most of the length of the Y chromosome, Y-STR loci are not statistically independent of one another (unlike standard autosomal STR markers) and are co-inherited as extended haplotypes of linked markers. Therefore, multiplication of single-locus allele frequencies to obtain estimated Y-STR haplotype frequencies is not appropriate. An estimation of the frequency of occurrence of a particular Y-STR haplotype necessitates the use of a counting method, which, with the limited sizes of databases available, produces an estimate that depends entirely upon the size of the database used. Thus, large databases of multi-locus Y-STR haplotypes need to be generated to maximize the probity of Y-STR evidence. A large comprehensive European-based Y-STR database is maintained by the Institute of Legal Medicine, Charité-University Medicine Berlin (www.yhrd.org).

However, although a subset of this database comprises the SWGDAM core loci, it is less useful for frequency estimates from haplotypes that have been generated using the two most popular commercial kits in the U.S., namely the PowerPlex®Y System(a,b) and AmpFlSTR®Yfiler™ PCR Amplification Kit. There are presently four online searchable Y-STR haplotype databases based in the United States and intended for forensic use. Three are maintained by commercial vendors: Reliagene, Inc., Promega Corporation and Applied Biosystems, Inc. The fourth is maintained by the University of Arizona. The National Center for Forensic Science (NCFS) also maintains a Y-STR database that will soon be available online. These databases differ in the number of Y-STR markers and individuals, although all possess the SWGDAM core loci. However, these

**Table 3.** Current U.S. - Based Y-STR databases.

| Agency | URL | Number of markers | Number of samples |
|---|---|---|---|
| National Center for Forensic Science | To be determined | 76 | 1.396 |
| University of Arizona | http:// Amadeus.biosci.arizona.edu/kcaldero/str | 83 | 2.518 |
| Applied Biosystems | www. Appliedbiosystems.com/yfilerdatabase | 17 | 3.561 |
| Promega Corporation | www.promega.com/techserv/tools/pplexy/ | 12 | 4.004 |
| Reliagen | www.reliagen.com/index.asp?menu_id=rdcontent | 11 | 4.623 |
| Potential Size of National Y-STR Database | | | 16.102 |

databases are somewhat limited in the number of individuals and loci profiled, which sometimes limits their operational usefulness. For example, the biggest U.S.-based database comprises haplotypes from 4,623 individuals. By combining data from these U.S. databases, a much larger Y-STR database of approximately 16,000 individuals can be created, resulting in a significant increase in the probative value of Y-STR evidence. Also, merging the NCFS and University of Arizona databases will increase the number of samples with extended Y-STR loci haplotypes, which may be of assistance to those interested in developing the next generation of Y-STR multiplex systems. Establishing a national database that incorporates data from a multitude of sources requires the implementation of a number of quality indicator metrics. Quality assurance procedures must be developed to govern the suitability and quality of data from diverse sources. For example, it may be necessary for donors of data to establish analytical prowess by testing externally provided proficiency samples. Since each commercial kit or academic multiplex system uses different primer sets, it will also be essential to ensure that allele calls are equivalent regardless of the multiplex system employed.

Importantly, merged data must be purged of duplicate samples that have been submitted by the same donor to multiple databases. To effectively manage the data, a Y-STR Database Consortium comprised of database stakeholders from commercial companies, academia, the FBI and U.S. crime laboratories was formed at the February 2006 AAFS meeting in Seattle (Table 3). It was agreed that NCFS, a program of the National Institute of Justice (NIJ) hosted by the University of Central Florida, would maintain and manage the consolidated Y-STR database on behalf of stakeholders. The National Institute of Justice is funding this effort. As a group, we are working to collate existing Y-STR data from various commercial and academic sources and have enlisted the aid of geographically diverse crime laboratories to furnish additional samples.

## MITOCHONDERIA

### Structure of mitochonderia

The typical human cell has several hundred mitochondria, cytoplasmic organelles that convert energy to forms that can be used to drive cellular reactions. Without them cells would be dependent on anaerobic glycolysis for all their adenosine triphosphate (ATP). The mitochondria have a characteristic double membrane structure, in which the outer membrane contains large channel-forming proteins (called porin) and is permeable to all molecules of 5000 daltons or less, while the inner membrane is impermeable to most small ions and is intricately folded, forming structures called cristae. The large surface area of the inner mitochondrial membrane accommodates respiratory chain and ATP synthase enzymes involved in the process of oxidative phosphorylation (OXPHOS). The mitochondrial matrix contains hundreds of enzymes, including those required for the oxidation of pyruvate and fatty acids and those active in the tricarboxylic acid (TCA) cycle. The matrix also contains several identical copies of the mitochondrial DNA, mitochondrial ribosomes, tRNAs and various enzymes required for the transcription and translation of mitochondrial genes (Alberts et al., 1994).

### Organization of the human mitochonderial genome

The human mitochondrial genome is 16,569 base pairs (bp) in length (Anderson et al., 1981), a closed, circular molecule located within the mitochondrial matrix and present in thousands of copies per cell. Mitochondrial DNA has two strands, a guanine rich heavy (H) strand and a cytosine-rich light (L) strand. The heavy strand contains 12 of the 13 polypeptide-encoding genes, 14 of the 22 tRNA-encoding genes and both rRNAencoding genes. Introns are absent in mtDNA, and all of the coding sequences are contiguous (Anderson et al., 1981; Wallace et al., 1992; Zeviani et al., 1996).

The only non-coding segment of mtDNA is the displacement loop (D-loop), a region of 1121 bp that contains the origin of replication of the H-strand (OH) and the promoters for L and H-strand transcription. The mtDNA is replicated from two origins. DNA replication is initiated at OH using an RNA primer generated from the L-strand transcript. H-strand synthesis proceeds two-thirds of the way around the mtDNA, displacing the parental H strand until it reaches the L-strand origin (OL), situated in a cluster of five tRNA genes. Once exposed on the displaced H- strand, OL folds a stem-loop structure

and L-strand synthesis is initiated and proceeds back along the H-strand template. Consequently, mtDNA replication is bidirectional but asynchronous (Clayton, 1982). MtDNA transcription is initiated from two promoters in the D-loop, PL and PH. Transcription from both promoters proceeds around the mtDNA circle, creating a polycistronic RNA. The tRNA genes which punctuate the larger rRNA and mRNA sequences then fold within the transcript and are cleaved out. The mRNAs and rRNAs liberated are posttranscriptionally polyadenylated and the tRNAs are modified and the 3' terminal CCA added (Attardi et al., 1982; Attardi and Montoya, 1983; Clayton, 1984; Wallace, 1992; Taanman, 1999).

## Mitochonderial DNA replication

MtDNA is replicated by the DNA polymerase gamma complex which is composed of a 140 kDa catalytic DNA polymerase encoded by the POLG gene and a 55 kDa accessory subunit encoded by the POLG2 gene (Van der Tovar, 2005). Replication of mitochondrial DNA begins on only one strand within the non-coding "control" region. As this strand is replicated, the opposite strand of the original DNA duplex is displaced and forms a single-stranded loop hence the name "D-loop" for Displacement loop. The displaced strand is then broken down and the replication process begins again displacing a single strand in the process (Schon et al., 2012).

## Reasons for using mitochonderial dna rather than nuclear dna

First, multiple copies: Each mitochondrion contains its own DNA, with many copies of the circular mitochondrial DNA in every cell. It is thought that each mitochondrion contains between 1 and 15, with an average of 4 to 5, copies of the DNA (Reynolds et al., 2000) and there are hundreds, sometimes thousands, of mitochondria per cell. The result is that there are many thousands of copies of the mitochondrial DNA in every cell. This compares with only two copies of nuclear DNA. Second, Better protection: The mitochondrion also has a strong protein coat that protects the mitochondrial DNA from degradation by bacterial enzymes. This compares to the nuclear envelope that is relatively weak and liable to degradation.

Third, higher rate of evolution: DNA alterations (mutations) occur in a number of ways. One of the most common ways by which mutations occur is during DNA replication. An incorrect DNA base may be added; for example, a C is added instead of a G. This creates a single base change, or polymorphism, resulting in a new form. These single base mutations are rare, but occur once in every 1,200 bases in the human genome. The result is that the rate of change, or evolutionary rate, of mitochondrial DNA is about five times greater than nuclear DNA (Bar et al., 2000). This is important in species testing, as even species thought to be closely related may in time accumulate differences in the mitochondrial DNA but show little difference in the nuclear DNA. Finally, maternal inheritance: A further reason for the use of mitochondrial DNA in species testing, and in forensic science, is its mode of inheritance. Mitochondria exist within the cytoplasm of cells, including the egg cells. Spermatozoa do not normally pass on mitochondria and only pass on their nuclear DNA. The resulting embryo inherits all its mito-chondria from its mother (Tully et al., 2004; Brown et al., 2002; Brown et al., 2000).

The result is that mothers pass on their mitochondrial DNA type to all their offspring, but only the daughters will pass on the mitochondrial DNA to the next generation. Mitochondrial DNA is therefore passed from generation to generation down the maternal line. Mechanisms for this include simple dilution (an egg contains 100,000 to 1,000,000 mtDNA molecules, whereas a sperm contains only 100 to 1000), degradation of sperm mtDNA in the fertilized egg, and, at least in a few organisms, failure of sperm mtDNA to enter the egg. Whatever the mechanism, this single parent (uniparental) pattern of mtDNA inheritance is found in most animals, most plants and in fungi as well. Also, most mitochondria are present at the base of the sperm's tail, which is used for propelling the sperm cells. Sometimes the tail is lost during fertilization. Also, unlike nuclear DNA, where there is a shuffling of the chromosomes at every generation, the mitochondrial DNA does not recombine with any other DNA type and remains intact from generation to generation (Brown et al., 2002; Brown et al., 2000; Pastores et al., 1994; Guntheroth et al., 1990). The role of DNA is to encode protein and RNA molecules, and the mitochondrial DNA is no different. All mammalian mitochondrial DNA is very similar, with the order and position of the genes being the same. The general structure of the mitochondrial DNA is shown in Figure 3. As with nuclear DNA, to indicate the significance of the match, analysts usually estimate the frequency of the sequence in some population. The estimation procedure is actually much simpler with mtDNA. It is not necessary to combine any allele frequencies because the entire mtDNA sequence, whatever its internal structure may be, is inherited as a single unit (a "haplotype"). In other words, the sequence itself is like a single allele, and one can simply see how often it occurs in a sample of unrelated people (Lutz et al., 2003; Alvarez et al., 2007).

This polymorphism allows scientists to compare mtDNA from crime scenes to mtDNA from given individuals to ascertain whether the tested individuals are within the maternal line (or another coincidentally matching maternal line) of people who could have been the source of the trace evidence (Coble et al., 2006).

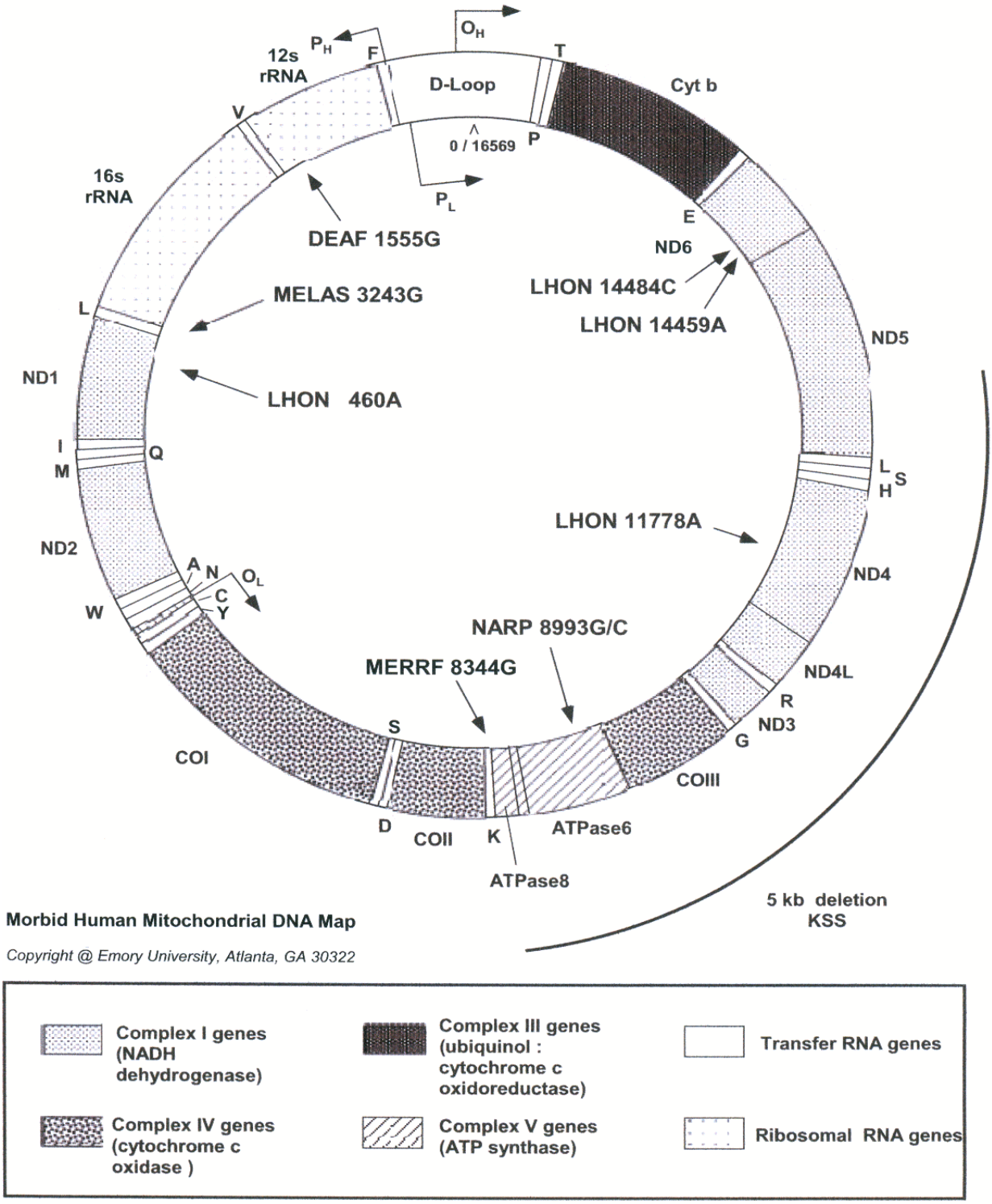


**Figure 3.** The Human Mitochondrial DNA Genome. The genes encoded by the mitochondrial DNA (mtDNA) genome are noted. Point mutations associated with mitochondrial diseases are noted in the center of the genome (Brown et al., 2002).

## Mitochonderial coding region as a source for variability

Forensic mtDNA analysis commonly investigates the HVI and HVII regions, but analysis of the coding region is gaining interest as this region harbours highly polymerphic mutations that may increase the discrimination of most common mtDNA types (Parsons and Coble et al.,

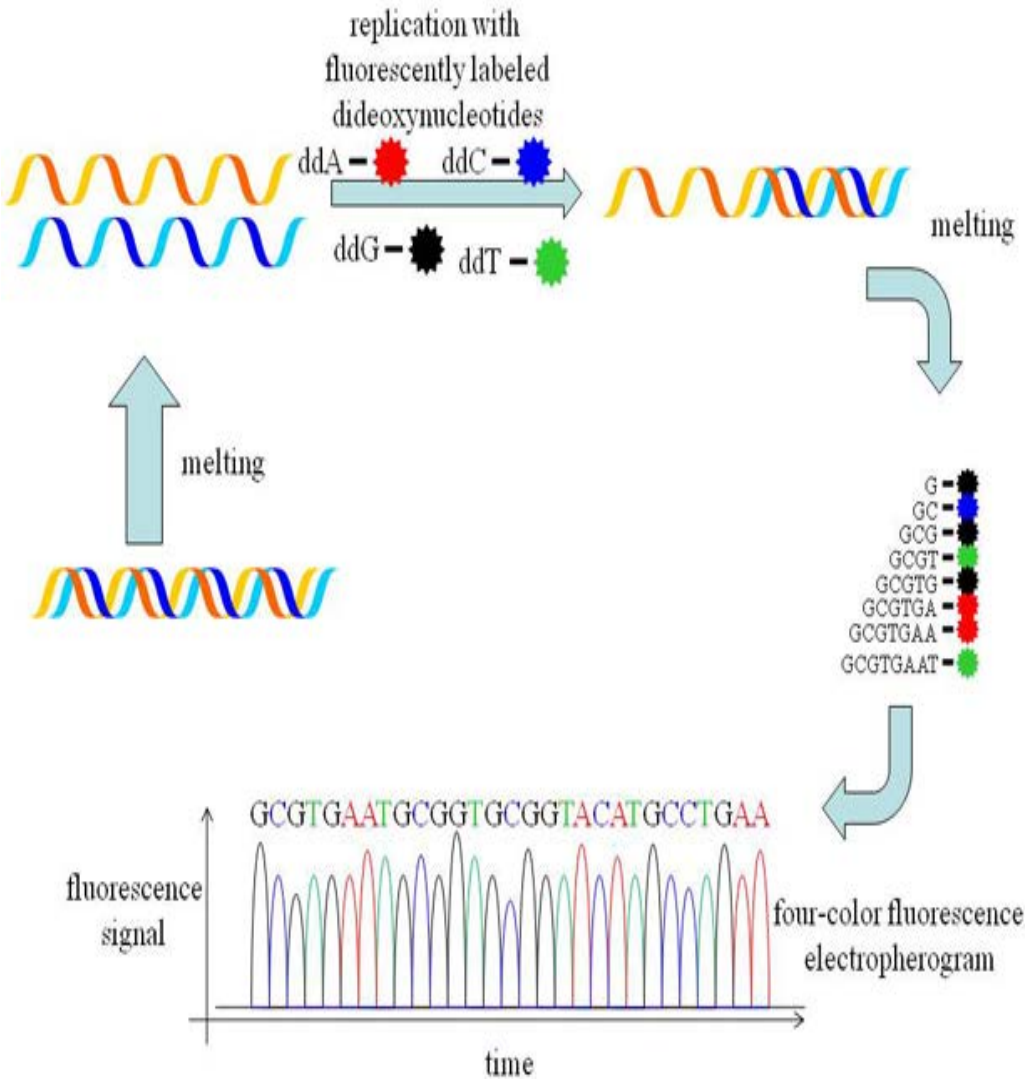


**Figure 4.** The sketch of Sanger capillary sequencing.

2001; Andreasson et al., 2002; Imad et al., 2014b). The development of hight throughput capillary sequencing has made whole mitochonderial genome analysis feasible and large collections of coding region data are now progressing (Herrnstadt et al., 2002). Thus, selection of new markers suitable for forensic coding mtDNA analysis is feasible. In a study by Coble et al. (2004) a penal of coding region SNPs were selected to resolve the most common HVI and HVII sequences including the H haplogroup. An SNP assay was chosen as whole mitochonderial genome sequencing is not a practical approach for the analysis of forensic samples. Moreover, coding SNPs were also chosen to resolve four additional commonly observed HVI and HVII haplotypes as the H-defining SNPs may not strained by only choosing silent and non-private polymorphism (variant observed) in more than one individual for ethical and practical reasons, nearly a four-fold increase in discrimi-nation of the five most common haplogroups (H, J, T, V and U) was obtained (Coble et al., 2004).

## INTERPRETATION OF SEQUENCE DATA

Current Sanger capillary sequencing systems are like the widely used Applied Biosystems (Figure 4), the dNTP/ddNTP mixture causes random, non-reversible termination of the extension reaction, creating from the different copies molecules extended to different lengths. Following denaturation and cleaning up of free nucleo-tides, primers, and the enzyme, the resulting molecules are sorted by their molecular weight (corresponding to the point of termination) and the label attached to the terminating ddNTPs is read out sequentially in the order created by the sorting step. Additionally, the advent of more sensitive detection systems and several rounds

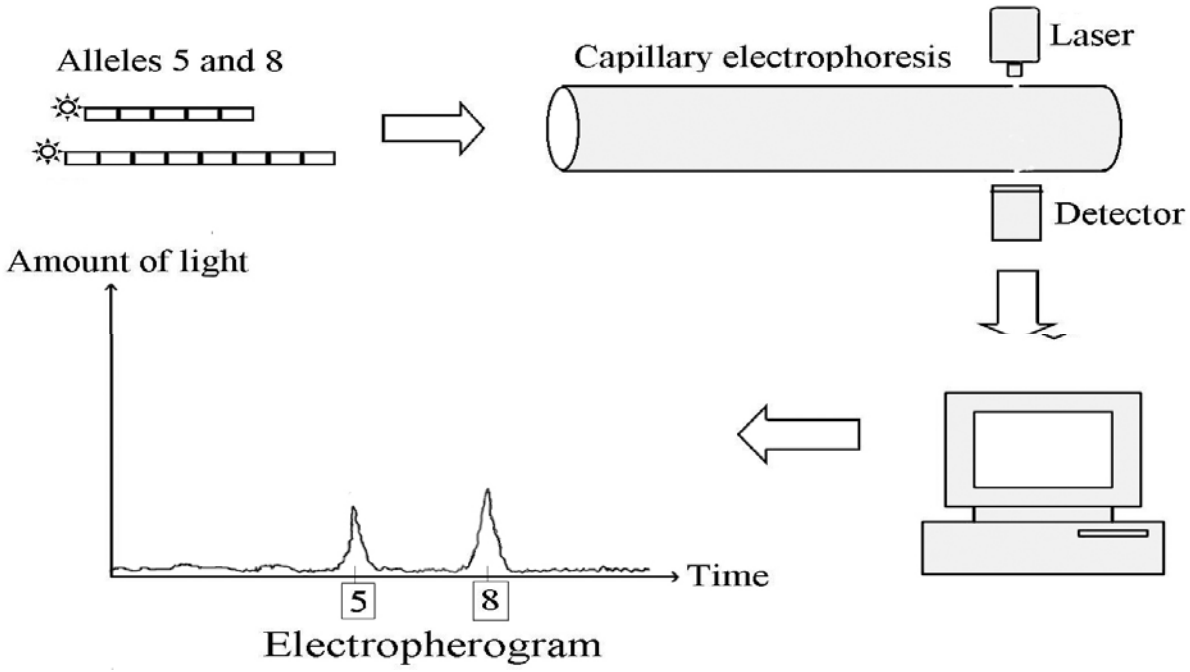


**Figure 5**. Shows the sketch of an electropherogram for two D16S539 alleles. One allele has eight repeats of the sequence GATA and the other has five .S small rectangle represents a GATA repeat. For illustration here only one copy of each allele (with a fluorescent molecule, or "tag" attached) is shown. However, PCR generates many more copies from the DNA sample with these alleles at the D16S539 locus. Whilst these copies are drawn through the capillary tube, the tags glow as the STR fragments move pass the laser beam. The colored light from the tags is measured using an electronic camera .A computer is finally used to produce the electropherogram based on the signals received.

of primer extensions (equivalent to a linear amplification) permit smaller amounts of starting DNA to be used for modern sequencing reactions. Using current Sanger sequencing technology, it is technically possible for up to 384 sequences (Emrich et al., 2002) of between 600 and 1,000 nt in length to be sequenced in parallel.

**Base designations:**

"A" designation—green peaks
"G" designation—black peaks
"T" designation—red peaks
"C" designation—blue peaks
"N" designation—peaks that, for whatever reason, are not clear enough to designate as A, G, T, or C. These bases are generally inconclusive. Often, a position that has an inconclusive (N) base in one direction may appear very clear in the other direction.

**TYPES OF POLYMORPHISMS**

Purines to purines or pyrimidines to pyrimidines - Transversions: (purines to pyrimidines or pyrimidines to purines).

Insertions: an extra base is present when compared to the Anderson reference sequence.
Deletions: a base is missing when compared to the Anderson reference sequence.

**SEQUENCHER™ 4.7**

The validate mitotypes command allows ours to compare the results of separate analysts. Select two contigs which have been assembled to the same reference sequence (SEQUENCHER™ 4.7 User Manual for Windows © 1991 - 2007 Gene Codes Corporation, Inc. All rights reserved).

After the raw sequencing data has been collected by the 310 genetic analyzer and analyzed by the Sequencing Analysis program, it must be reviewed manually and interpreted with the aid of the Sequencher™ software (Figure 5). Sometimes the data will present characteristics that make interpretation more complex, though not impossible. Interpretive skills improve with experience, but listed below are suggestions for those less familiar with mtDNA analysis on dealing with some of the more common features that affect interpretation. However, all data (especially indications of mixtures, heteroplasmy, etc.) should be reviewed on a case-by case basis, and interpretations made

by the analyst based on his or her own expertise.

The forensic community generally recognizes the HV1 region as consisting of base positions 16024-16365 and HV2 as base positions 73-340, as numbered according to the Cambridge Reference Sequence (also referred to as the Anderson Sequence) (Bainbridge et al., 2011). This laboratory will attempt to sequence all the bases within these defined regions, as well as an additional 25 base pairs in either direction (that is, 15999-16390 and 48-365). While it is preferable to have confirmation of each sequence by comparing the forward and reverse strands, it is sometimes necessary to use two forward strands or two reverse strands as confirmation of the sequence (Al-Haggar et al., 2013; Pareek et al., 2011). In these cases it is suggested that the sample be cycle sequenced a second time to obtain the confirmatory strand in the same direction. In addition to case samples, a positive and negative control must be sequenced. The purpose of controls is to show that each stage of the analysis is working properly. Therefore, only one positive and negative control is necessary for each step of the procedure (for example, if samples from three amplification runs are combined into one cycle sequencing run, only one positive and one negative must be carried through to show the sequencing reagents and thermal cycler have performed as expected). If the results obtained for the controls are not as anticipated, the evaluation of the results will be determined on a case-by-case basis (Goldberg et al., 2006).

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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*Full Length Research Paper*

# Distribution and molecular detection of apple mosaic virus in apple and hazelnut in Turkey

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**Apple mosaic virus (ApMV) is one of the most important diseases limiting the production of hazelnut and apple in Turkey and the objectives of this research were to determine the convenient and reliable method for RNA isolation and also to determine primer pair for real time polymerase chain reaction (RT-PCR) detection of coat protein gene for Turkish ApMV isolates. Apple mosaic virus isolates were collected in 2007 to 2010 and the presence of the pathogen was detected by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and RT-PCR tests. Six different RNA extraction protocols and three primer pairs were applied in RT-PCR amplifications and 44 hazelnut and 15 apple ApMV isolates were obtained. All of the amplicons were subjected to enzymatic digestion with restriction endonuclease enzymes and phylogenetic analysis were performed according to the digestion profiles.**

**Key words:** Apple mosaic virus, coat protein gene, hazelnut.

## INTRODUCTİON

Apple (*Malus domestica* L.) is a pome fruit widely distributed and produced in Turkey. Turkey is the fourth largest producer world-wide (Koksal et al., 2010), supplying about 3.5% of the world's production. The major cultivated varieties are Starking Delicious, Golden Delicious, Granny Smith, Fuji and some local varieties such Amasya, Tokat etc. Hazelnut (*Corylus avellana*) is native to Turkey and Turkey is the world leader in hazelnut production (Koksal et al., 2010). One of the most important factor that limits apple and hazelnut production in Turkey is *Apple mosaic virus* (ApMV, family Bromoviridae, genus *Ilarvirus*). The virus is primarily pruning (Brunt et al., 1996). Major hosts of the virus are transmitted by vegetative propagated material and hops, horse chesnut, hazelnut, raspberry, birch, rose, some herbaceous plants (Rybicki, 1995) and strawberry (Tzanetakis and Martin, 2005). The virus causes symptoms such as severe systemic mosaic in apple, mottle, oak leaf patterns and ringspots on the hazelnut trees. The symptoms are evident early in the growing season but they may become partly masked with time and as temperatures increase on hazelnut trees (Kobylko and Nowak, 2006). The virus is also transmitted by root bridges between trees. The virus also causes great reduction in the size and the yield of hazelnut (Gentit et al., 2009). Control of graft-transmissible viruses includes

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**Table 1.** Primers used for RT-PCR amplification of ApMV coat protein gene targets.

| Primer | Reference | PCR product (bp) | Ann. temp. (°C) |
|---|---|---|---|
| Sense: 5'-TCA ACA TGG TCT GCA AGT AC-3'<br>Antisense: 5'-CTA ATC GCT CCA TCA TAA TT-3' | Lee et al. (1998) | 680 | 54 |
| Sense: 5'-ATC CGA GTG AAC AGT CTA TCC TCT AA-3'<br>Antisense: 5'-GTA ACT CAC TCG TTA TCA CGT ACA A-3' | Menzel et al. (2002) | 262 | 62°C |
| Sense: 5'- GGC CAT TAG CGA CGA TTA GTC- 3'<br>Antisense: 5'- ATG CTT TAG TTT CCT CTC GG-3' | Petrzik and Lenz (2002) | 820 | 50°C |

sanitation, quarantine and certification programs but the success of those programs depends on specific and sensitive methods for pathogen detection. The most effective way of controlling viral infections in woody plants is establishing virus-free plantations with virus-tested planting material.

This research was conducted to investigate the suitable RNA extraction method and the primer pairs work with both RNAs in RT-PCR analysis of capsid protein (CP) gene of ApMV and also to determine the genetic variability on the basis of restriction analysis of apple and hazelnut CP genes since those genes are frequently used in genetic variability (Petrzik and Lenz, 2002).

## MATERIALS AND METHODS

### Virus source

Hazelnut samples were collected from different varieties from the Eastern and Western Black Sea Region of Turkey (Duzce, Sakarya, Samsun, Ordu, Giresun and Trabzon provinces) in 2007 to 2010. Each plant sample was taken per 1,000 $km^2$ area of plantation. Apple samples were çollected from a number of varieties in the major apple production areas (Ankara, Antalya-Korkuteli, Tokat, Nevşehir, Konya, Nigde, Isparta provinces) with the same procedure. Young leaves, bark tissues and fruits were collected from symptomatic and asymptomatic plants and kept in deep-freezer at -20°C until they were processed.

### ELISA procedure

Standard DAS-ELISA procedure was applied to all of the apple and hazelnut specimen collected with commercial antiserum (Agdia), according to manufacturer's instructions. Young leaves were subjected to the test.

### RNA extraction

Six different RNA extraction procedures were applied to both ApMV infected plant tissues. Young bark scraps and leaf tissues of hazelnut and apple leaves were used for RNA extraction. Six different RNA extraction methods were applied to both specimens: 1) Spiegel et al. (1996) method, 2) Menzel et al. (2002) method, 3) Rott and Jelkman (2001) method, 4) Zhang et al. (1998) method, 5) Verwoerd et al. (1989) method and 6) QIAGEN RNeasy Plant mini kit. Total plant RNAs were isolated by using silica in the procedures of Menzel et al. (2002); Rott and Jelkman (2001), ds-RNA's were isolated in the procedure of Zhang et al. (1998) and then subjected to RT-PCR. After total RNAs were extracted, they were quantified by Nanodrop 2000 (Thermo Scientific).

### Primers and PCR mix for RT-PCR

One step RT-PCR was performed using the primers of the coat protein gene of ApMV shown in Table 1. PCR mix was organised and used throughout the RT-PCR analysis. It was as follows: virus specific primers (up and down stream) (0.2 µM each), 1.5 mM dNTP mix (Fermentas), 2.5 µl 10 X Taq buffer, 8 u MMLV-RT (Fermentas), 1.2 u RNase inhibitor (Promega), 0.6 µM $MgCl_2$, 1 U Taq DNA Polymerase (Fermentas), 2 µl RNA template. Total reaction volume was 25 µl.

### RT- PCR amplification

PCR protocols were applied according to the thermocycling conditions of each technique and for Menzel et al. (2002), it was as: 30 min at 42°C, 15 min at 95°C and 34 cycles of 30 s at 94°C, 30 s at 62°C and 1 min at 72°C, amplification was finalised by 7 min at 72°C. Amplified fragments of ApMV CP genes were all subjected to electrophoresis in 1% agarose slab gels at 100 V for 1 h and then stained with 0.5 $\mu g ml^{-1}$ of ethidium bromide.

### Phylogenetic analysis

Restriction enzymes used for digestion of RT-PCR amplified products were Hinf*I* (Genemark), *BamHI* (Genemark), Ava*I* (Genemark), *EcoRI* (Sigma), Ssp*I* (Fermentas), Rsa*I* (Fermentas), *HindIII* (Genemark), and Hinc*II* (Genemark). PCR products (8 µl), 1 µl enzyme buffer and 0.5 µl of specific restriction enzyme were incubated overnight at 37°C and then subjected to gel electrophoresis in 6% polyacrylamide at 100 V for 2 h. Gels were stained with ethidium bromide and visualised by Bio-imaging system (Gene Genius) (Ulubas et al., 2009). Phylogenetic analysis has been performed according to the restriction profiles, by using Jacord's similarity index programme of Numerical Taxonomy and Multivariate Analysis System, Version 2 programme (Rohlf, 1998).

## RESULTS

### Virus source

During the surveys performed between 2007 to 2010, a

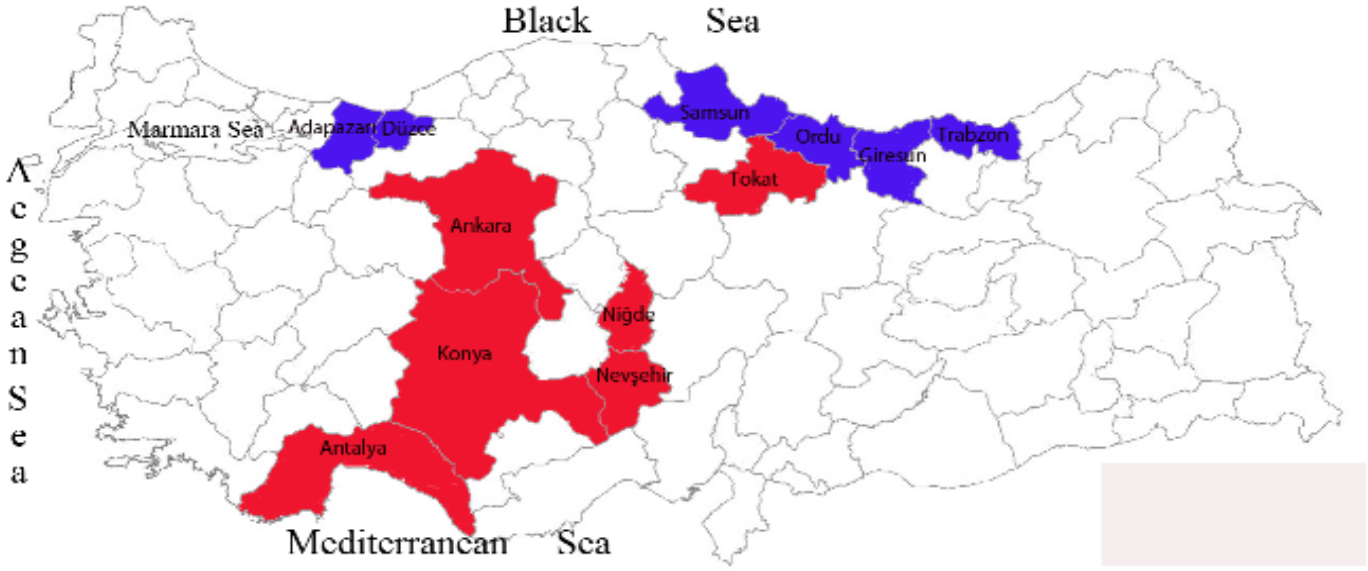


**Figure 1.** The survey area (areas shown in blue are the provinces surveyed for hazelnut and areas shown in red are the provinces surveyed for apple).

**Figure 2.** The systemic mosaic symptoms of ApMV on hazelnut (top) and apple (bottom) trees.

total of 194 hazelnut and 47 apple specimens were collected from the survey area as shown in Figure 1. Since the infection can be masked in arid climates, both symptomatic and asymptomatic specimens were collected from the research area. Major symptoms of virus infection in hazelnuts were chlorotic oak leaf pattern mosaic, vein clearing and diffuse ringspots whereas the symptoms observed on apples were severe systemic mosaic (Figure 2). The symptomatic plants were commonly present at the side rows of hazelnut whereas symtomatic plants were usually in the middle of the apple orchards. The infection was present only on Granny Smith variety of apples but in the case of hazelnut, it was common on local varieties; such as, Karafindik, Tombul, Giresun yaglisi, Sivri, Foca, Mincane, Palaz, Cakildak and Badem. The virus was present along all the Black Sea Coast.

## DAS-ELISA

According to DAS-ELISA results, ApMV infections were detected in 44 out of the 194 of hazelnut specimens (22%) and in 15 out of 47 apple specimens (31.9%). All of the ApMV positive isolates were symptomatic and showed symptoms typically associated with infection by ApMV as shown on Figure 2. The ApMV infection was present in all of the provinces surveyed for apple. Crop loss was greater in hazelnut production than in apple and the infection caused almost no yield. Although, ELISA is routinely used for detecting the plant viruses, this technique is not sufficient to detect the low concentrations of the virus that occur in some tissues or

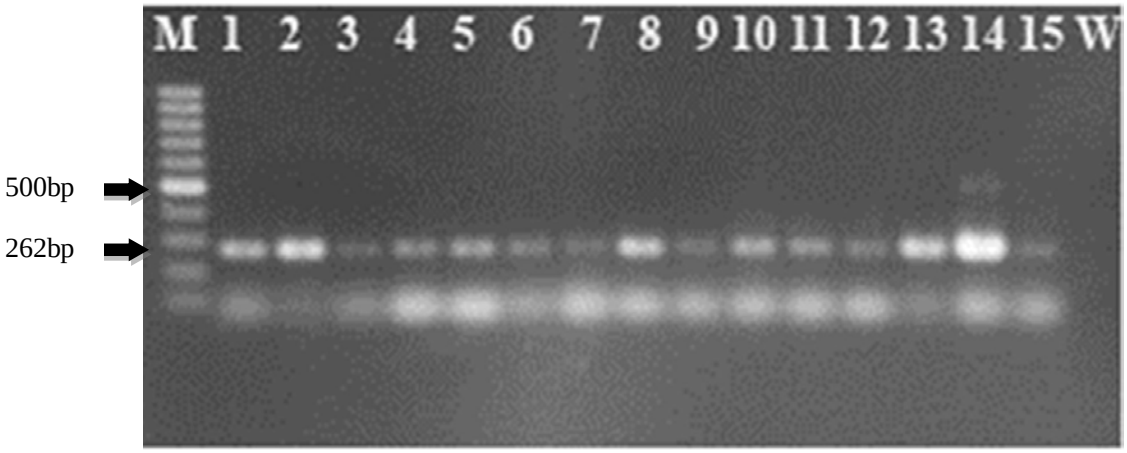


**Figure 3.** RT-PCR amplification of Turkish ApMV apple isolates. M, GeneRuler 100bp DNA ladder(Fermentas); 1, Ankara; 2, Antalya-Korkuteli; 3-5, Isparta-Eğirdir; 6, Isparta-Eğirdir BKAE (fruit); 7-8, Isparta-Gelendost; 9, Isparta-Gelendost (fruit); 10 Isparta-Gelendost; 11-12, Tokat, 13-15, Nevşehir isolates.

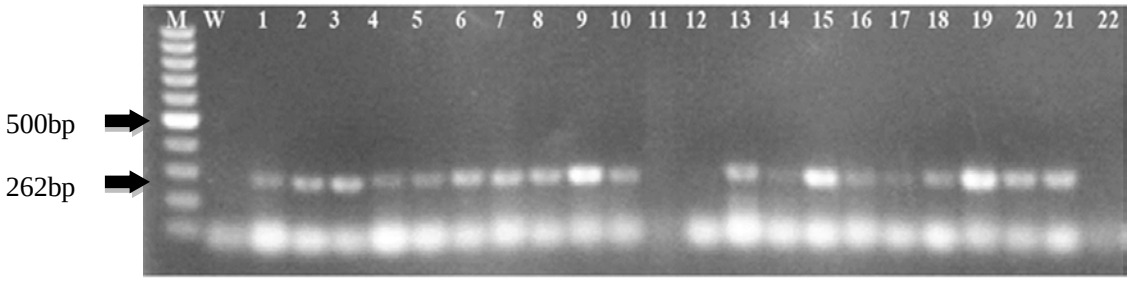


**Figure 4.** RT-PCR amplification of Turkish ApMV hazelnut isolates. M, GeneRuler 100bp DNA ladder, Fermentas; 1-3, Düzce; 4-11; Adapazarı; 12-17, Giresun; 18-19, Ordu; 20-21, Giresun isolates; 22, water control.

in some hosts. ELISA may fail because of the inhibitory effects of polysaccharides or phenolic compounds in tissue extracts of woody plants, thus ELISA seems inappropriate with dormant woody tissues (Menzel et al., 2002; Kobylko and Nowak, 2006; Akbas and Değirmenci, 2010). Some of the hazelnut isolates showing the typical symptoms of infection were detected as negatives by ELISA but were then detected as positives in RT-PCR amplification. Therefore, ELISA alone is not sufficient for a reliable detection of ApMV from the infected hazelnut tissues. The infection was not present in asymptomatic apple and hazelnut trees.

## RNA extraction

Extracted RNAs were amplified only with the Menzel et al. (2002)'s primers for coat protein gene of ApMV (the same primer pair was used to amplify both apple and hazelnut RNAs) and the amplified products of RT-PCR were 262 bp long fragments (Figures 3 and 4). PCR mix used in this research gave better results with hazelnut and apple extracted RNAs in RT-PCR amplification compared to the original PCR mix of Menzel et al. (2002). RNA isolation from infected hazelnut specimens is known to be problematic because of the high tannin content and uneven distribution of the pathogen in the infected tissues (Kobylko and Nowak, 2006) so this problem was overcomed by using the RNA extraction procedure of Rott and Jelkman (2001) for hazelnut specimens. The yield and purity of hazelnut RNA extracts obtained using Rott and Jelkman's procedure was quite high compared to the other procedures tested, 3.5 to 4 µg per 100 mg fresh tissue and 260/280 ratios were between 1.7 to 1.9 and quality and amount of the RNA extracts obtained from bark scraps were better comparing the leaf tissues.

## RT- PCR amplification

The extracted RNAs were amplified only with Menzel et al. (2002)'s primer pair and no amplification was obtained with the other primer sets tested. Some RNAs obtained from apple fruits were amplified and showed clear bands

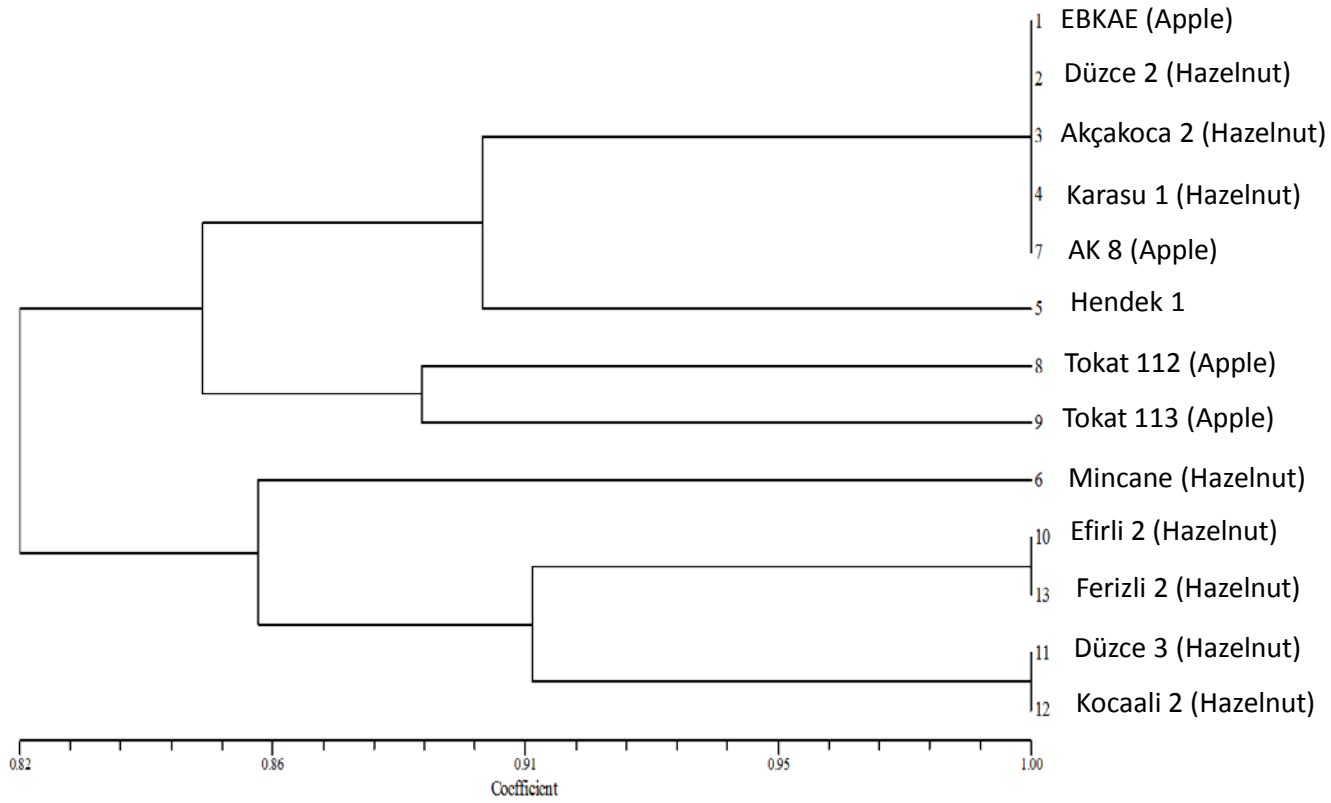


**Figure 5.** Dendogram of Turkish *Apple mosaic virus* isolates.

on gels whereas hazelnut fruits were generally empty or small and very weak amplification bands were obtained from those samples. Therefore, leaves and fruits of apple and bark of young branches of hazelnut were preferred for total RNA extraction. Among the primers tested, only amplifications were obtained with Menzel et al. (2002)'s primer set. RNA extracts of hazelnut flowers were also amplified with the same primer pair (Akbas and Degirmenci, 2010). Our research demonstrated that optimization of the PCR protocol by suitable additives to the PCR mix, reduced the adverse affects of the plant polysaccharides and phenolics. Hazelnut bark tissues and apple leaf tissues were shown to be good templates for reliable detection and amplification of ApMV in one step RT-PCR. As a result of RT-PCR procedure, 44 hazelnut and 15 apple isolates were amplified and produced very clear and sharp bands as shown in Figures 3 and 4.

### Phylogenetic analysis

In order to detect genetic polymorphism between the capsid protein genes of isolates of ApMV from hazelnut and apple, all of the RT-PCR products were subjected to enzymatic digestion with restriction endonuclease enzymes of Ava*I,* Bam H*I*, Hinf*I*, Ssp*I* EcoR*I*, Hinc*II*, Hind*III* and *Rsa*I. Ava*I*, Bam H*I*, Hinf*I* and Ssp*I* enzymes digested the RT-PCR products whereas the other enzymes (EcoR*I*, Hinc*II*, Hind*III* and Rsa*I*) had no effect on the amplified product and failed to digest them. The present results demonstrate that genotypic variation of Turkish ApMV isolates were quite high, at 85% similarity; four clusters were obtained in phylogenetic analysis (Figure 5). In the first cluster, two apple and three hazelnut isolate collected from Western Black Sea Region were similar and settled in the same cluster. The second cluster consisted only 2 apple isolates collected from the same province (Tokat). Third cluster is consisting of hazelnut isolates collected from Eastern Black Sea Region and the fourth cluster is consisting of hazelnut isolates of Western Black Sea Region.

## DISCUSSION

The results of the present study indicates that ApMV can be detected by one step RT-PCR and it is possible to extract total RNAs from leaf tissues of apple and also dormant tissues of hazelnut although the tissues contain high amount of phenolic compounds and tannins. In Turkey, ApMV is a widespread pathogen and has been detected previously in hazelnut (Akbas et al., 2004), apple (Ulubas and Ertunc, 2003) cherry (Gumus et al.,

2008), and rose tissues (Yardimci and Culal, 2009) by ELISA. In Poland, ApMV was detected only in Negret and Gustav Zellemus cultivars among the 49 cultivars tested by ELISA (Piskornik et al., 2002; Kobylko and Nowak, 2006). Furthermore, it was not possible to obtain reliable RNA extraction from hazelnut tissues because of the high tannin and phenolic compounds. Investigators have tried to overcome this problem by inoculation to an herbaceous host such as *Phaseolus vulgaris* (Sokmen, 2003). Recently, Akbas and Degirmenci (2010) have isolated total RNA only from flower tissues of hazelnut and Ertunc et al. (2011) analysed Turkish and Ukranian ApMV apple isolates and compared them for coat protein composition. We succeeded to detect ApMV from the leaves and dormant tissues of hazelnut and apple trees within this work by using the same primer pair. The ability to detect ApMV in hazelnut and apple tissues will provide a valuable tool for certification programs and this protocol can easily be incorporated into the testing protocols of quarantine for rapid screening of imported mother plants of apple and screening of national collections of hazelnut in Turkey.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

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African Journal of Biotechnology

*Full Length Research Paper*

# Preparation of chitosan nanoparticles for loading with NPK fertilizer

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**In this paper the preparation of chitosan nanoparticles was carried out using methacrylic acid (MAA) and studied by both ultraviolet (UV)- visible transmission spectrophotometry and transmission electron microscopy (TEM). Nanoparticles with sizes as small as 17 to 25 nm were achieved. The obtained nanoparticles had a homogeneous morphology showing a quite uniform particles size distribution with a spherical shape. The solution was pH-sensitive, due to swelling and aggregation of the nanoparticles. The use of slow release fertilizer has become a new trend to save fertilizer consumption and to reduce environmental pollution. In this work, chitosan (CS) nanoparticles were obtained for the loading of NPK fertilizers. The stability of the CS-PMAA colloidal suspension was higher with the addition of nitrogen and potassium than with the addition of phosphorus, due to the higher anion charge from the calcium phosphate than the anion charges from the potassium chloride and urea. The mean diameter increase of the CS-PMAA nanoparticles in suspension with the addition of different compounds was P > K > N which indicates that the elements are being aggregated and loaded on the surface of the chitosan nanoparticles.**

**Key words:** Nanoparticles formation, chitosan, fertilizers, nanosolution.

## INTRODUCTION

The evolution of nanotechnology and the emergence of novel nanodevices and nanomaterials open up new trends in agriculture and biotechnology. Nanoparticles are molecules that are so small to fall within the nanometric range, with at least one of their dimensions being less than a few hundred nanometers. This small size rather brings about marvelous changes in their physical properties with respect to those observed in bulk materials. There are a large variety of methods that can be used in the production of nanoparticles, using techniques from both physics and chemistry. Among the first ones, arc-discharge, high-energy ball milling, laser pyrolysis and laser ablation are the most commonly used. Electrochemical and chemical vapour deposition, sonochemistry and different wet chemistry routes (for example, sol-gel, co-precipitation, inverse micelles, etc.) are also widely employed (Malendi et al., 2008).

Preparation of nanoparticles is recently under heavy

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**Abbreviations: N,** Nitrogen; **P,** phosphorus; **K,** potassium; **CS,** chitosan; **PMAA,** poly methacrylic acid.

research because of its various applications, in food processing, biomedical, optical, and electronic devices (de Moura et al., 2008). The usage of natural polysaccharides in the preparation of nanoparticles has attracted attention because of their biodegradability and hydrophilic characters which are favorable characters in multiple applications. Chitosan nanoparticles emerged due to their unequalled properties (de Moura et al., 2008).

Fertilizers are chemical compounds applied to enhance plant growth (Bahera and Panda, 2009; Corradini et al., 2010). Usually, fertilizers are applied either through the soil or by foliar spreading. Artificial fertilizers are inorganic compounds devised in appropriate concentrations and combinations to supply three main nutrients: nitrogen, phosphorus and potassium (N, P and K) for multiple crops and growing conditions (Corradini et al., 2010). N (nitrogen) induces leaf growth and forms proteins and chlorophyll. P (phosphorus) enhances root, flower and fruit development. K (potassium) induces stem and root growth and synthesis of proteins (Mandal et al., 2009).

Saigusa (2000) pointed out that 40 to 70% of nitrogen, 80 to 90% of phosphorus, and 50 to 70% of potassium of the normal fertilizers are lost to the environment and not absorbed by plants, causing intrinsic economic and resource losses and dangerously increasing environmental pollution. Recently, the usage of slow release fertilizers has become a new tendency to save fertilizer consumption and to reduce environmental pollution (Wu and Liu, 2008; Corradini et al., 2010). This presents the idea of developing encapsulated fertilizers in which NPK fertilizers are loaded within nanoparticles (Teodorescu et al., 2009). Thus, the fertilizers are preserved by the nanoparticles for better survival in inoculated soils, permitting their controlled release into the soil (Saigusa, 2000). Therefore, the procedure of loading of fertilizers in polymeric nanoparticles is relatively novel, with multiple commercial applications.

Chitosan nanoparticles have been investigated as a carrier for drug delivery, meanwhile a few studies have been attempted to discover the potential of chitosan nanoparticles as controlled release for NPK fertilizers. Chitosan is a polysaccharide derived from chitin, which may be obtained from crustaceans (Boonsongrit et al., 2006). Several preferred properties have been studied, such as film-forming ability, gelatinous characteristics and bio-adhesion.

Due to its polymeric cationic charac-teristics, chitosan may react with negatively charged molecules and polymers, showing a favorable reaction (Boonsongrit et al., 2006). The ability of controlling nanoparticle size is highly favorable for most applications in nanotechnology (de Moura et al., 2008).

Chitosan nanoparticles fabricated via different preparation protocols have been in recent years widely studied as carriers for therapeutic proteins and genes with varying degree of effectiveness and drawbacks. In general, it was demonstrated that the polyionic coacervation process for fabricating protein loaded with chitosan nanoparticles offers simple preparation condi-tions and a clear processing window for manipulation of physiochemical properties of the nanoparticles (for example size and surface charge), which can be conditioned to exert control over protein encapsulation efficiency and subsequent release profile. The weakness of the chitosan nanoparticle system lies typically with difficulties in controlling initial burst effect in releasing large quantities of protein molecules (Gan and Wang, 2007).

Naturally occurring polymers, such as chitosan, have been extensively studied as carriers for therapeutic protein and gene delivery systems. β-Lactoglobulin (β-LG) is a member of the lipocalin superfamily of transporters for small hydrophobic molecules. Agudelo et al. (2013) examined the binding of milk β-Lactoglobulin with chitosan of different sizes such as chitosan 15, 100, and 200 KD in aqueous solution at pH 5 to 6, using FTIR, CD, and fluorescence spectroscopic methods.

In their study, structural analysis showed that chitosan binds β-LG via both hydrophilic and hydrophobic contacts with overall binding constants of $K_{\beta\text{-LG-ch-15}}$ = 4.1 (±0.4) × $10^2$ $M^{-1}$, $K_{\beta\text{-LG-ch-100}}$ = 7.2 (±0.6) × $10^4$ $M^{-1}$, and $K_{\beta\text{-LG-ch-200}}$ = 3.9 (±0.5) × $10^3$ $M^{-1}$ with the number of bound protein per chitosan (*n*) 0.9 for ch-15, 0.6 for ch-100, and 1.6 for ch-200. Chitosan 100 KD forms stronger complexes with β-LG than chitosans 200 and 15 KD. Polymer binding did not alter protein conformation inducing structural stabilization. Chitosan 100 is a stronger protein transporter than chitosan 15 and 200 KD (Agudelo et al., 2013).

Biodegradable chitosan of different sizes were used to encapsulate antitumor drug doxorubicin (Dox) and its *N*-(trifluoroacetyl) doxorubicin (FDox) analogue (Sanyakamdhorn et al., 2013). In their study, the structural analysis showed that Dox and FDox bind chitosan via both hydrophilic and hydrophobic contacts with overall binding constants of $K_{\text{Dox-ch-15}}$ = 8.4 (±0.6) × $10^3$ $M^{-1}$, $K_{\text{Dox-ch-100}}$ = 2.2 (±0.3) × $10^5$ $M^{-1}$, $K_{\text{Dox-ch-200}}$ = 3.7 (±0.5) × $10^4$ $M^{-1}$, $K_{\text{FDox-ch-15}}$ = 5.5 (±0.5) × $10^3$ $M^{-1}$, $K_{\text{FDox-ch-100}}$ = 6.8 (±0.6) × $10^4$ $M^{-1}$, and $K_{\text{FDox-ch-200}}$ = 2.9 (±0.5) × $10^4$ $M^{-1}$, with the number of drug molecules bound per chitosan (*n*) ranging from 1.2 to 0.5. The order of binding is ch-100 > 200 > 15 KD, with stronger complexes formed with Dox than FDox.

The molecular modeling showed the participation of polymer charged $NH_2$ residues with drug OH and $NH_2$ groups in the drug–polymer adducts. The presence of the hydrogen-bonding system in FDox-chitosan adducts stabilizes the drug-polymer complexation, with the free binding energy of -3.89 kcal/mol for Dox and -3.76 kcal/mol for FDox complexes. The results showed that chitosan 100 KD is a more suitable carrier for Dox and FDox delivery (Sanyakamdhorn et al., 2013).

The objectives of this study were to obtain chitosan nanoparticles by polymerizing methacrylic acid for the

loading of NPK fertilizers. The reaction and stability of chitosan nanoparticles suspension containing N, P and K were estimated by UV- visible transmission spectrophotometry and TEM.

## MATERIALS AND METHODS

Chitosan (CS) (MW 71.3 kDa, degree of deacetylation 85%) was purchased from Sigma-Aldrich (Germany). All reagents were of analytical grade. Potassium persulfate ($K_2S_2O_8$) and methacrylic acid (MAA) were purchased from Sigma-Aldrich (Germany). Calcium phosphate ($Ca(H_2PO_4)_2{\cdot}H_2O$), urea ($CO(NH_2)_2$) and potassium chloride KCl were purchased from Sigma Chemical Co. (St. Louis, USA).

### Preparation of CS-PMAA nanoparticles

As recommended by de Moura et al. (2008) and Corradini et al. (2010), the CS-PMAA nanoparticles were obtained by polymerizing MAA in CS solution in a two-step process. In the first step, 0.2 g chitosan was dissolved in methacrylic acid aqueous solution (0.5%, v/v) for 12 h under magnetic stirring.

In the second step, with continued stirring, 0.2 mmol of $K_2S_2O_8$ was added to the solution, until the solution became clear. The polymerization was subsequently carried out at 70°C under magnetic stirring for 2 h which leads to the formation of CS-PMAA nanoparticle solution, which was thereafter cooled in an ice bath.

### Description of CS-PMAA nanoparticles

The morphology and size of the CS-PMAA nanoparticles were investigated using a JEOL 1010 transmission electron microscope at 80 kV (JEOL, Japan). One drop of the nanoparticle solution was spread onto a carbon-coated copper grid and was subsequently dried at room temperature for transmission electron microscopy (TEM) analysis. The sizes of the nanoparticles were determined directly from the figure using an Image-Pro Plus 4.5 software. The value is an average size of three parallels.

The percentage transmittance of CS-PMAA nanoparticle solution was measured using UV-visible spectrophotometer in the wavelength range from 330 to 730 nm. The readings were recorded and plotted graphically.

### Loading of NPK fertilizer in chitosan nanoparticles

The sources of N, P and K used were urea, calcium phosphate, and potassium chloride, respectively. These substances were used separately. The loading of NPK fertilizers in chitosan nanoparticles was obtained by dissolving suitable amounts of NPK into 100 ml of CS-nanoparticle solution under magnetic stirring for 8 h at 25°C. The following concentrations: i) 500 ppm of N; ii) 60 ppm of P and iii) 400 ppm of K were finally obtained in each solution.

The maximum P solution concentration was of 60 ppm because the solution precipitates at higher concentrations. The resulting solutions had a pH of 4.5.

### Measurement of zeta potential of CS-PMAA and CS-PMAA-NPK nanoparticles

The zeta potential measurements of CS and CS-NPK nanoparticles were taken on a Zetasizer NanoZS (Malvern Instruments, Worcestershire, UK). The measurements were carried out after equilibrating the prepared samples at pH 4.5 at 25°C. All analyses were performed in triplicate.

## RESULTS

Figure 1 shows a transmission electron microscopy (TEM) photo of the chitosan nanoparticles (CS-PMAA). These nanoparticles showed a rather spherical shape with a homogeneous size distribution. The mean diameter of CS nanoparticles was of approximately 20±2 nm. Figure 2 shows the UV-visible transmittance spectra of the CS-PMAA nanoparticles. The interaction between CS and PMAA may be elucidated. The CS-PMAA species presented different values at the wavelength range (from 330 nm to 580 nm) reaching a maximum value of 83.4% at 630 nm.

Careful examination of Figures 3, 4 and 5 indicates that all nanoparticles accumulated presented a homogenous morphology with regular particle size distribution and a spherical shape. TEM micrographs of nanoparticles of CS-PMAA either alone or in combination with nitrogen, potassium or phosphorus show that the mean diameter of the CS-PMAA nanoparticles in suspension was approximately 20±2 nm and increased with the addition of the different compounds. The percentage increase in the mean diameter was of 54% with the addition of phosphorus of 31% with the addition of potassium and of 19% with the addition of nitrogen. Figure 6 shows the difference in zeta potential of CS-PMAA nanosolution and the other solutions with N, P and K at pH 4.5.

## DISCUSSION

During the preparation of chitosan nanoparticles, it was observed that the chitosan solution in methacrylic acid (MAA) changed from a clear to a semiturbid suspension. This conversion is an evidence of the formation of chitosan nanoparticles with MAA. According to the mechanism proposed by de Vasconcelos et al. (2006), the formation of nanoparticles occurs via inter and intramolecular linkages between PMAA carboxyl groups and amino groups of chitosan during the process of polimerization of MAA.

CS molecules in solution are in cationic electrolytic form, which tends to make the formation of specific structures via electrostatic reactions with MAA easy and leads to the makeup of CS-PMAA nanoparticles through polymerizing MAA in the presence of CS (de Moura et al., 2008; Zhang et al., 2010). This is in agreement with results for similar systems using acrylic acid reported in the literature (de Vasconcelos et al., 2006). It is known that the interplay of other reactions may also contribute to the stability of systems formed by CS and PMAA, such as hydrophobic association of methyl groups proceeding (Chen et al., 2007; de Moura et al., 2008).

CS-PMAA nanoparticles were formed by the reaction

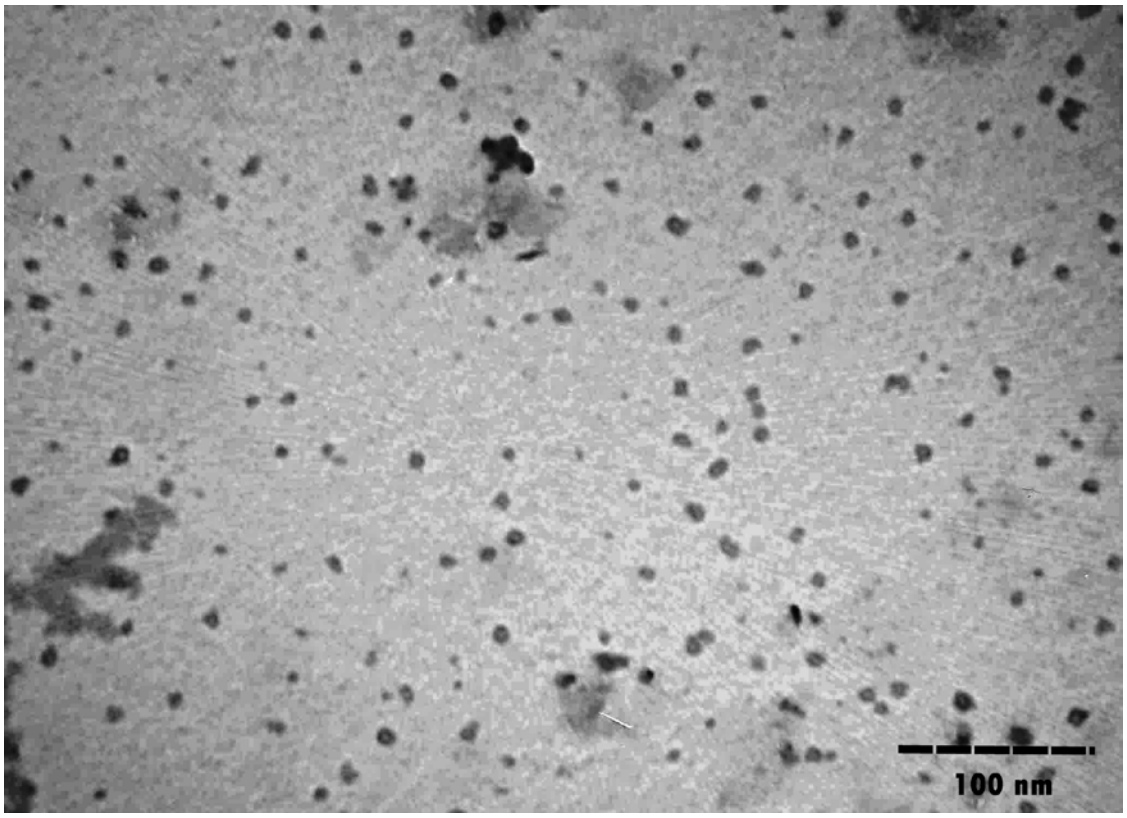


**Figure 1.** TEM microphotograph obtained for chitosan nanoparticles (CS-PMAA) at pH 4.5.

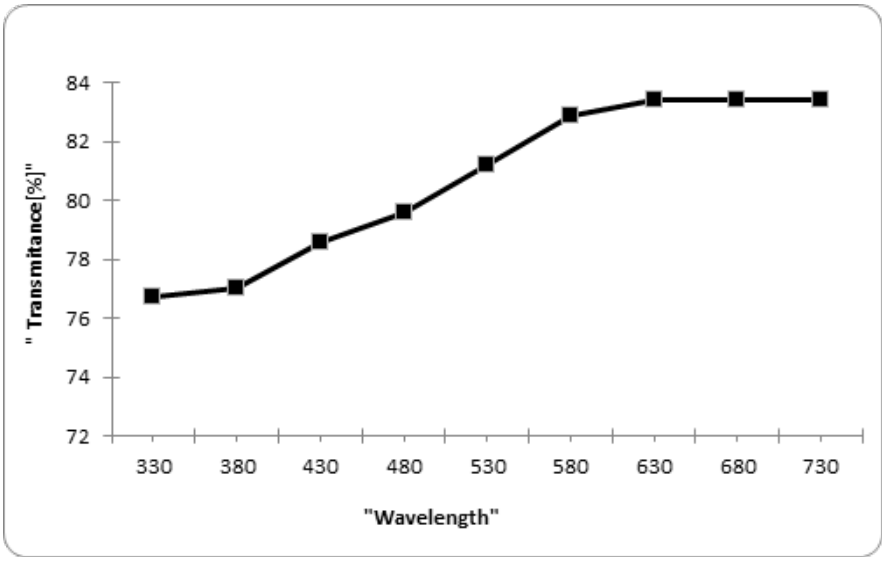


**Figure 2.** Percentage transmittance of CS-PMAA nanosolution.

between positively charged CS and negatively charged PMAA (de Moura et al., 2008). These results are very interesting, since they do not only show that CS-PMAA nanoparticles are pH sensitive, but that by controlling the pH, their surface may be charged either positively or negatively, which is of key value to know the solution stability state as well as for their applications (Wu et al., 2006; de Moura et al., 2008).

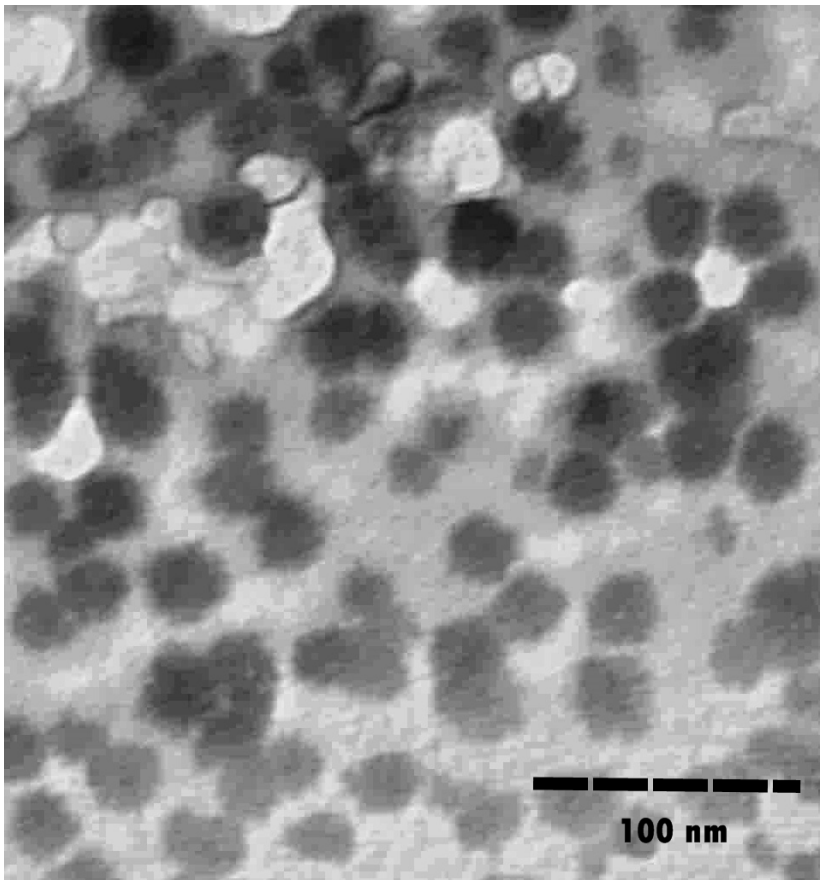


**Figure 3.** TEM micrograph obtained for chitosan nanoparticles loaded with potassium (K).

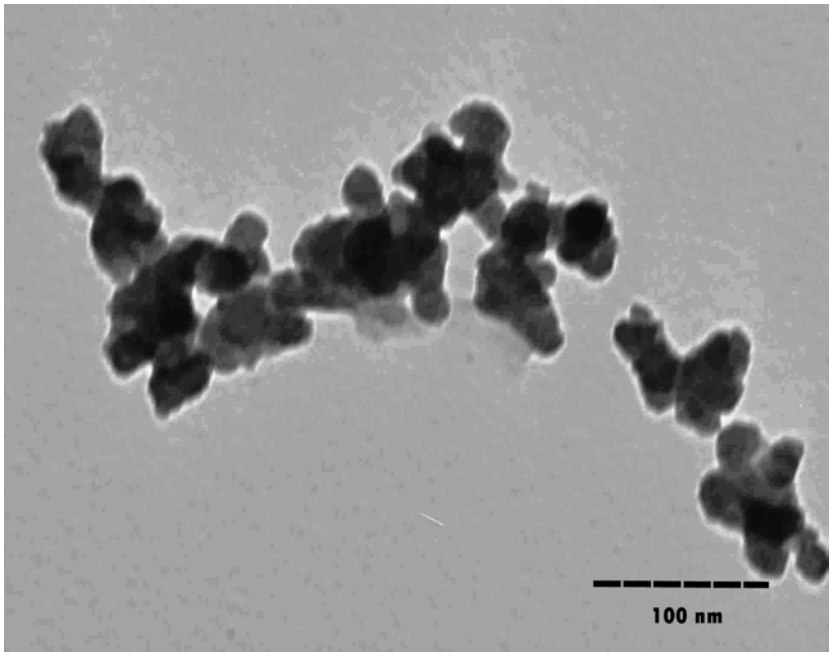


**Figure 4.** TEM micrograph obtained for chitosan nanoparticles loaded with nitrogen (N).

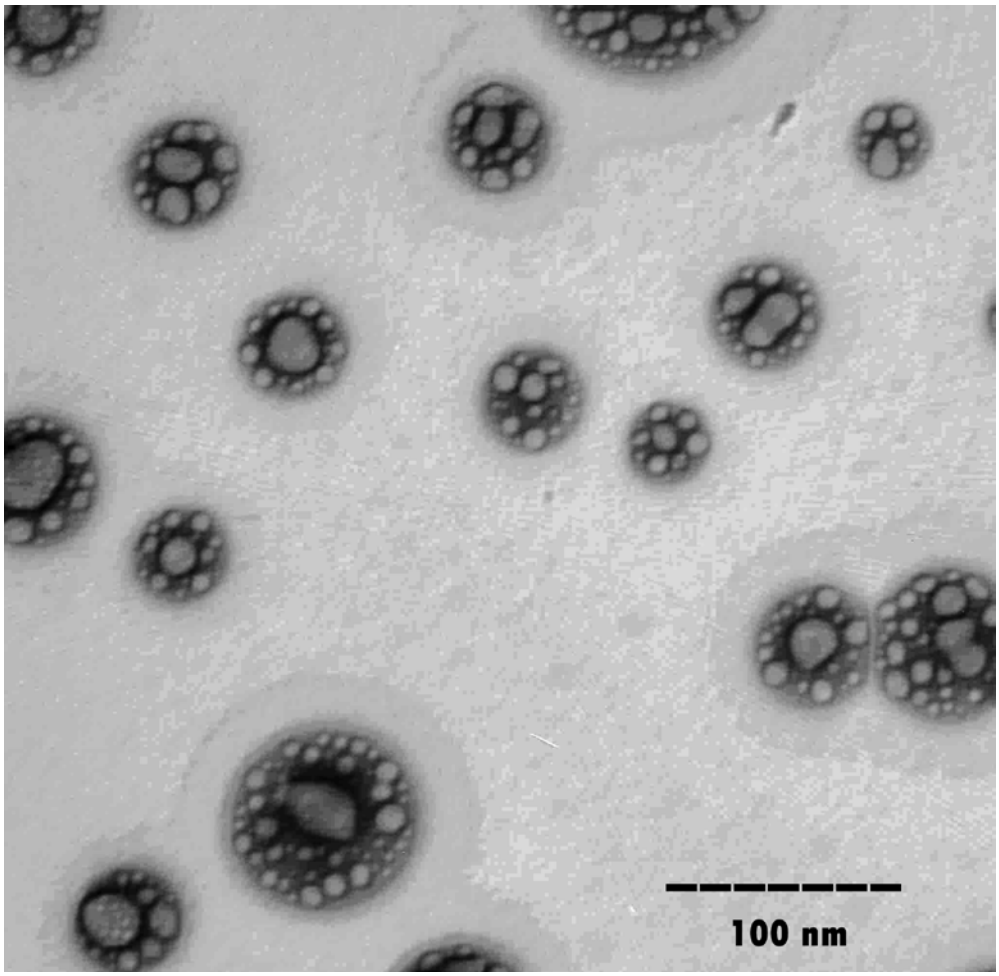


**Figure 5.** TEM micrograph obtained for chitosan nanoparticles loaded with phosphorus (P).

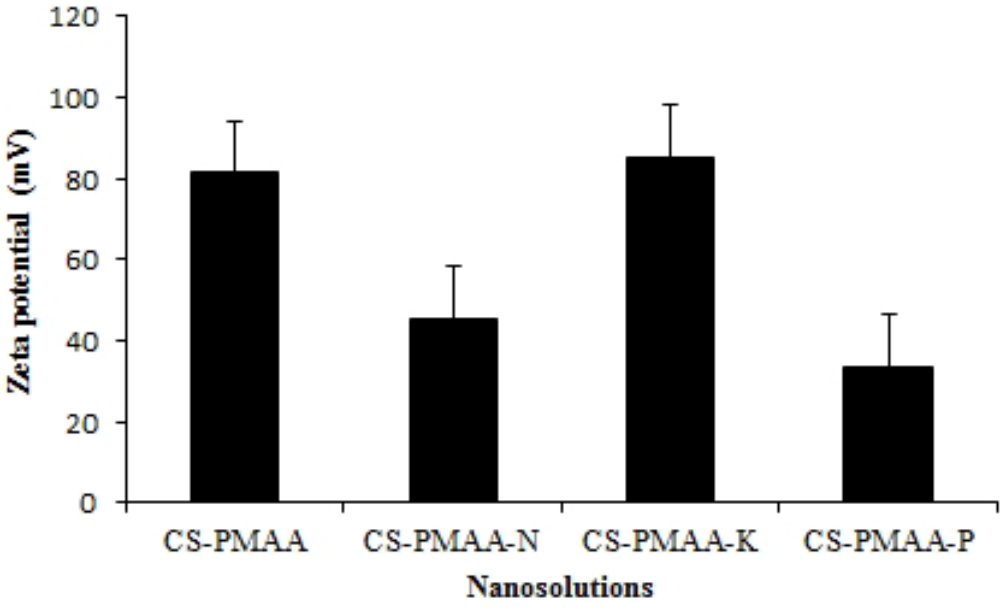


**Figure 6.** Zeta potential of CS-PMAA and CS-PMAA-NPK nanosolutions.

The values of percentage increase in nanoparticle diameter (listed above in the results section) are related to the size of the species separated in the colloidal suspension, and species separated from the urea have smaller value than the accumulated species from the KCl and calcium phosphate. These results are not in accordance with what had been reported in Corradini et al. (2010) study with regard to potassium and nitrogen.

The stability of a colloidal dispersion is remove as a result of the electrical double layer on the surface of the colloidal particles. When an electrolyte is added to a colloidal dispersion, it suppresses the diffused double layer and reduces the zeta potential. This reduces the electrostatic repulsion between the particles and precipitates the colloid. The colloid is so sensitive to ions of opposite sign. A positively charged colloidal dispersion is precipitated by negative ions, these ions are incorporated into the fixed portion of the double layer, reducing the total charge of the particle and the reverse to this is true. The ion with the same charge of the colloidal particle has little effect on precipitation.

The highest Zeta potential value was recorded for CS-PMAA- K solution, that of 85.4 mV which indicates the higher stability of the solution in comparison with that of N (45.3 mV) and that of P (33.6 mV). The zeta potential of the K-nanosolution was higher than that of the chitosan nanosolution (81.3 mV) which proves the high stability of the resulting nanosolution of K. It was found that for the colloidal dispersions of CS-PMAA with phosphate, the addition of 60 ppm of phosphate makes the stability of the colloidal system under the conditions studied reaches a maximum. The dispersions of CS-PMAA combined with 500 ppm of N were higher in stability compared with that of phosphorus. Corradini et al. (2010) showed that above 500 ppm of N a reduction of positive charges is occurring in the colloidal dispersion of CS-PMAA, which is due to the presence of negative groups from the urea molecules. For dispersion with potassium, the stability of solution is confirmed with the addition of 400 ppm. This shows that the presence of $Cl^-$ ions (from KCl) did not affect the stability of colloidal dispersion with the addition of up to 400 ppm (Wu et al., 2006; de Vasconcelos et al., 2006; de Moura et al., 2008; Corradini et al., 2010).

Further studies are needed to understand the mechanism and to optimize the loading of the N and P elements into the CS-PMAA nanoparticles to reach rather stable solutions and to study the effects of nanofertilizers and their applications on plant growth.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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*Full Length Research Paper*

# Association mapping of resistance to Verticillium wilt in *Gossypium hirsutum* L. germplasm

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**Verticillium wilt is a major disease affecting the growth of cotton. For screening the resistant genes, 320 *Gossypium hirsutum* germplasms were evaluated in Verticillium nursery, and association mapping was used to detect the markers associated with the Verticillium wilt resistance. 106 microsatellite marker primer pairs were used to estimate the genetic diversity, population structure and linkage disequilibrium (LD) of the germplasm. Polymorphism (PIC) was found to be 0.53, and population structure were detected to be three subgroups (K=3). LD decay rates were estimated to be 13 to 15cM ($r^2$ ≥0.20). Significant associations between polymorphic markers and Verticillium wilt resistance traits were observed using the general linear model (GLM) and mixed linear model (MLM). Four loci showed positive effects on the phenotype which meant that these loci could promote the Verticillium wilt resistance of cotton, and thirteen loci showed negative effects in GLM. The results displayed that association mapping could complement and enhance quantitative loci (QTLs) information for marker-assisted selection in cotton breeding.**

**Key words:** Cotton germplasm, verticillium wilt, simple sequence repeats (SSR) markers, linkage disequilibrium (LD), association analysis.

## INTRODUCTION

Verticillium wilt is one of major diseases that has been found in most cotton cultivated areas and caused severe yield losses in the popular field. This disease is induced by the soil-borne fungus *Verticillium dahliae* Kleb that can survive in the soil for long periods of time (Wilhelm, 1955). In view of incurability for fungicides, breeding and using of disease-resistant cultivars are the mainly available method of protecting cotton from the infection of pathogen. Understanding the genetic events of this disease at the molecular level will help us to utilize existing resistance in cotton germplasm for breeding. The genes of resistance to Verticillium wilt were firstly considered to be recessive according with the model of Mendel in the populations of G. *hirsutum* (Brinkerhoff and

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Ashagari,1970; Roberts and Staten, 1972). Ma et al., (1996) and Zhang et al., (2000) concluded that the resistance to Verticillium wilt was controlled by a dominant gene. However, Devey and Roose (1987) and Wang et al., (2004) deduced a conclusion that the resistance to Verticillium wilt was quantitative trait and was controlled by minorgene. Quantitative loci (QTLs) of Verticillium wilt resistance were detected in G. barbadense and in G. hirsutum in the past years (Yang et al., 2007, 2008, 2009; Jiang et al., 2009), some important genes have been cloned (Simko et al., 2004).

Molecular markers or QTLs linked with important traits have been identified in many crops based on the linkage analysis of F2- , RIL-, or DH (double haploid)-derived mapping populations using molecular marker technology. However, some important QTLs might not be detected because of linkage disequilibrium (LD) in mapping and breeding populations. Association mapping could be valuable for validating and detecting more QTLs in complex-pedigree population relevant linkage analyses. This method has been successfully used in different crops to identify markers and genes associated with a variety of phenotypes based on the nature population. *tb1* was the first gene that was found to be associated with short branches of maize using the approach of association mapping in plant (Doebley et al., 1997; Wang et al., 1999; Jaenicke-Després et al., 2003). Then, a large number markers were discovered to be associated with kernel size in wheat (Breseghello and Sorrels, 2006), heading date and water-stress tolerance in barley (Kraakman et al., 2004; Ivandic et al., 2003), resistance to late blight in potato (Gebhardt et al., 2004; Karolina et al., 2009), and fiber quality in *G. hirsutum* (Abdurakhmonov et al., 2008, 2009).

Association mapping was usually affected by the population structure (Pritchard et al., 2000a). False markers would be detected because of populations which were composed of individuals deriving from a complex pedigree. The use of population structure could significantly correct the number of false positives in plant studies (Thornsberry et al., 2001). Currently, STRUCTURE was the main software to evaluate the population structure (Pritchard et al. 2000b), especially the population of allogamy plants. In this study, we analyzed the association of simple-sequence repeat (SSR) markers with resistance to Verticillium wilt in a collection of cotton germplasm from all over the world, and aimed to provide theory basis and feasible method for the molecular marker-assisted selection.

## MATERIALS AND METHODS

### Plant material

For the association analysis, 320 *G. hirsutum* germplasm were used. These cotton germplasm were collected from all over the world and conserved in the Gene Bank of Cotton Research Institute at Chinese Academy of Agricultural Sciences. All these cotton germplasm have been strictly self-pollinated during the past years.

### Phenotype evaluation

*V. dahliae* VD race were isolated from the soil of local field in Anyang which was moderate pathogenic and defoliating (ND) pathotypes. Then the soil of nursery was inoculated with *V. dahliae* cotton seed cultivation.

These cotton germplasm were grown in the disease nursery of Cotton Research Institute at Chinese Academy of Agricultural Sciences. Each individual was sown in one row with three repeats and randomized completely in different blocks. One line named Jimian 11 that was susceptible to Verticillium wilt was used as the control cultivars, which was usually used in the evaluation of Verticillium wilt resistance (Wu et al.,1999; Du and Zhou, 2005). The plant spacing was maintained at 0.7×0.30 m. The damage of leaf and vascular tissue in seedlings and maturity stage were classified into five grades, which was the national standard of evaluation of Verticillium wilt resistance in China (Wu et al.,1999). The grades scored as 0, 1 and 2 were considered as resistance to Verticillium wilt, and grades 3 and 4 as susceptibility to Verticillium wilt. The index of damage was calculated as Wu et al. described (1999): $DI = \sum( d_i \times n_i ) / ( n_t \times 4 ) \times 100$, $d_i$ was the grade of damage, $n_i$ was the number of seedlings in the corresponding grade of damage, $n_t$ was the total seedlings. When the damage index of sensitive cultivar was about 50, the damage index of other individuals must be evaluated as soon.

### Genotyping with SSR markers

DNA was extracted from the young and fully expanded leaves of each species (Paterson et al., 2003). The sequences of SSR primers were downloaded from CMD (Cotton Marker Database, www.cottonmarker.org/cgi-bin/panel.cgi). Polymorphic SSRs were screened from a standard panel including upland cotton cultivars and other tetraploid species (Blenda et al., 2006). 106 SSRs were selected from the polymorphic primers. PCR reacted in 10 μL volumes included 1.0 μL10×Buffer (consisting of 20 mM $MgSO_4$, 100 mM KCl, 80 mM $(NH_4)_2SO_4$, 100 mM Tris-HCl, pH 9.0, 0.5% NP-40), 50 ng template DNA, 0.5 mM dNTP, 0.4 units of *Taq* DNA polymerase, 0.5μM forward and reverse primers. The PCR amplification program included 3 min pre-denature at 95□C, 30 cycles of 94°C 45 s, 57°C 45 s, 72°C 1 min, and 7 min extension at 72°C. The reactions were completed by PTC-100TM thermocycler. The PCR product was stored at 4°C before being run on the 8% non-denature PAGE gel (Sambrook et al., 1992). The gel was dyed by referring to Zhang et al.'s method (2000), and then was photographed using SYNGENE gel system.

### Allele diversity and population structure

When markers produced a single band, each allele was scored with "1". Whereas markers produced more than two bands, alleles were scored with "1", "2", "3"and "4" representing the numbers of bands, respectively. The missing data was represented with "-9". Diversity and heterozygosity were calculated based on 106 polymorphic SSR data in 320 lines. Allele frequencies were calculated using SpaGeDi ver.1.3 software (Hardy and Vekemans, 2002). The polymorphic information content (PIC) was analyzed using the PowerMarker 3.25 software package (Liu and Muse, 2005). The genetic distance (GD) was estimated using Neighbor Joining (N-J) algorithms with the minimum evolution objective function.

**Table 1.** Summary statistics of verticillium resistance index.

| Parameter | Mean | Standard deviation | Minimum | Maximum | P-value of normality test[a] | Q1[b] | Median | Q3[c] |
|---|---|---|---|---|---|---|---|---|
| Verticillium resistance index | 37.46 | 10.45 | 10.41 | 76.91 | 0.97 | 29.51 | 36.08 | 44.61 |

[a] Shapiro–Wilk test, [b] Quantile 25%, [c] Quantile 75%

**Table 2.** Mean squares of the ANOVA of verticillium resistance index.

| Parameter | Degree of freedom (d.f.) | Verticillium resistance |
|---|---|---|
| Cultivar | 321 | 328.08* |
| Replicate | 2 | 29.61 |
| Error | | 53.74* |
| $R^2$ of model | | 0.75 |

* indicate significance at the probability levels of 0.001.

#### Population structure analysis

Bayesian was estimated using STRUCTURE software for the population structure analysis (Pritchard et al., 2000b). The number of populations tested was assumed as K where K varied from 1 to 10. The length of running time was 100 000, and replication after burning was 10 000 for the STRUCTURE with admixture model. However, we did not find distinct clusters and could not determine a significant number of K populations using STRUCTURE. So we built a graph of Pn to find a proper value of K following the method of Evanno et al. (2005).

#### Linkage disequilibrium

LD parameter $r^2$ was estimated using Tassel 2.1 software (http://www.maizegenetics.net). LD between all pairs of SSR alleles was analyzed with MAF filtered datasets, where SSRs alleles with a 0.05 frequency in genotyped accessions were removed before conducting LD analyses, because minor alleles are usually problematic and biased for LD estimates between pairs of loci (Mohlke et al., 2001; McRae et al., 2001). The MAF removal was performed using the TASSEL site filtration function. LD was estimated by a weighted average of squared allele-frequency correlations between SSR loci. The significance of pairwise LD (p-values≤0.005) among all possible SSR loci was evaluated using TASSEL with the rapid permutation test in 10 000 shuffles. The LD values between all pairs of SSR loci were plotted as LD plots using TASSEL to estimate the general view of genome-wide LD patterns and evaluate 'block-like' LD structures.

#### Association studies

The general linear model (GLM) association test was performed after incorporating index of damages, SSRs genotype, and Q matrix using the TASSEL 2.1 software (Bradbury et al., 2007). The Q of population was set as covariate, and 1 000-time permutations were set for the correction of multiple testing. The Q matrix was created with K = 3 as determined by STRUCTURE. The phenotypic allele effect was estimated using the method described by Breseghello and Sorrells (2006): $a_i = \sum x_{ij} / n_i - \sum N_k / n_k$ where $a_i$ was the phenotype effect of specific i allele, $x_{ij}$ was the phenotype value of j individual with i allele, $n_i$ was the total individuals with i allele, $N_k$ was the phenotype value of j individual with null i allele and $n_k$ was the total individuals with null i allele.

## RESULTS

### Morphological traits

Phenotype values of Verticillium wilt resistance showed a wide range variation, and revealed that the data of traits was favorable for the association analysis (Table 1, Table 2). The lowest value was 10.40 meaning that the individual was the most resistant to the Verticillium wilt. The highest value was 76.9 presenting that the individual was the most sensitive.

### Diversity and structure

In the population, 106 SSR markers detected 278 loci and 333 SSR alleles with an average of 3.1 alleles per marker (from 2 to 6 alleles) (Table 3); whereas the average effective alleles were 2.4 varying from 1.2 to 4.9. The polymorphic information content (PIC) was calculated, and the average PIC of the population was found to be 0.53 ranging from 0.17 to 0.79. The genetic distance (GD) was estimated using the NTSYSpc Version 2.1, and the average GD was got to be 0.26 ranging from 0.04 to 0.57, which demonstrated the significant ranges of genetic diversity of the population.

The structure of the population was estimated with the

**Table 3.** Summary of SSR polymorphisms.

| Number of polymorphic SSRs | | | | Polymorphic information content (PIC) | | Genetic distance | |
|---|---|---|---|---|---|---|---|
| Locus | Average allele/ marker | Effective Allele | Rare Allele (%) | Range | Average | Range | Average |
| 278 | 3.1 | 2.4 | 23 | 0.17-0.79 | 0.53 | 0.04–0.57 | 0.26 |

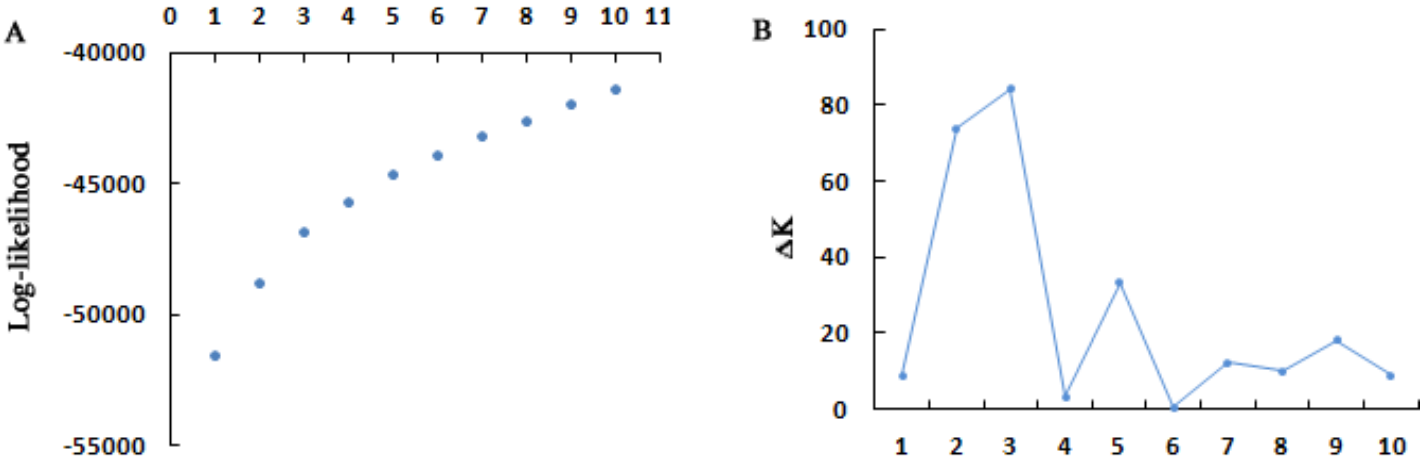


**Figure 1.** Analysis of the population structure. The numbers of subgroups were calculated using STRUCTURE (Pritchard et al. 2000b); A: Graph about the log-likelihood. The log-likelihood increased with the number of groups (K) increasing. B: Graph about **Δ**K. ΔK= m(|L(K + 1) − 2 L(K) + L(K − 1)|)/s[L(K)] was used to assess the number of groups (K) (Evanno et al. 2005). A clear peak was detected for K =3.

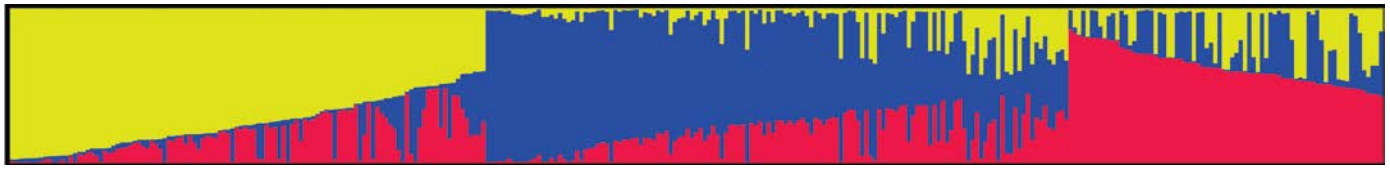

**Figure 2.** Population structure and cluster analysis: The bar plots of Q-matrix estimates for the variety accessions: Groups were represented in different colors (Blue for group 1, Green for group 2, Red for group 3).

STRUCTURE 2.3. The most appreciable value of K was found to be K = 3 after calculating the second-order change in log-likelihood described by Evanno et al. (Figure 1). Thus, 320 lines were assigned to three sub-populations, which were presented as three different bar plots (Figure 2). The bar plots showed that some individuals owned at least 50% of the single ancestral genetic background. So these individuals were assigned to three subgroups that were consisted of 101, 126 and 63 lines separately and were labeled as group1, group 2 and 3, respectively. The remaining 30 lines that showed a probability lower than 50% were assigned to a mix group.

## Linkage disequilibrium analysis

The LD of the population was estimated using Tassele2.1 for the accurate analysis of association. 11.6 percent of SSR loci pairs were found to be linkage disequilibrium ($p \leq 0.01$, $r2 \geq 0.01$), where 10.9 percent of loci pairs were found to be decayed over the long terms of cotton cultivar selection (38503 pairwise comparisons).

LD parameter $r^2$ was estimated for the calculation of the LD decated rate, and haplotypic LD was studied in the genome with 278 loci covering most of the chromosomes. It was found that most of $r^2$ ranged from 0.0 to 0.1, and

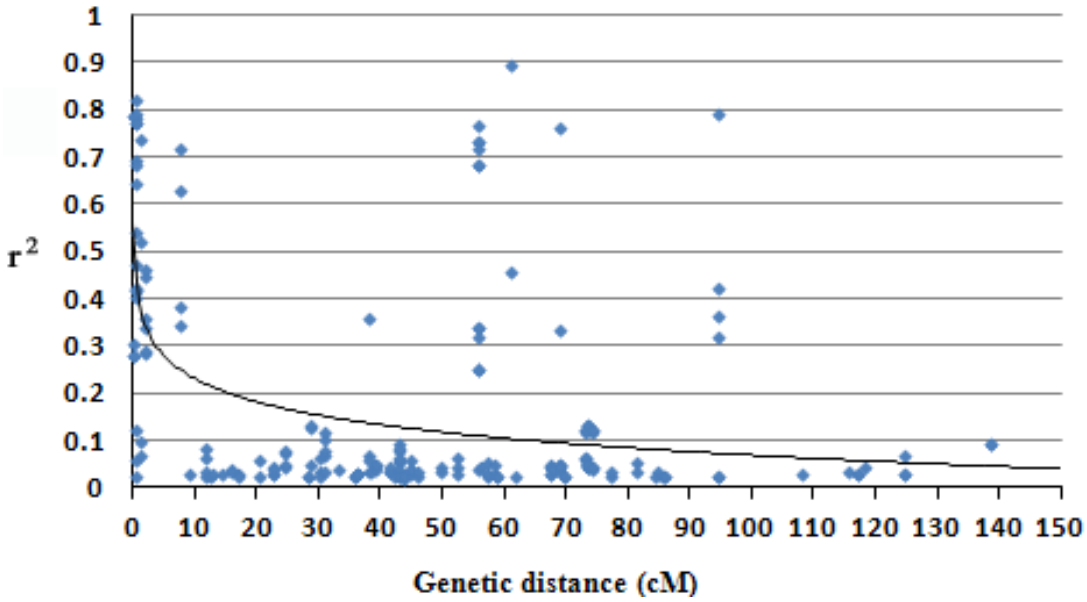


**Figure 3.** The decays of linkage disequilibrium by $r^2$ against genetic distance between all pairs of SSR loci. LD-decay is considered at the threshold of $r^2 \leq 0.2$ based on trend line.

most of LD ($r^2 \geq 0.2$) located in the distance of less than 15 cM. These results meant that the LD of the population decayed with the genetic distance (Figure 3).The LD decay rate was usually measured using the chromosomal distance when $r^2$ dropped to half its maximum value, and was used to evaluate the level of the LD decay. In this study, the LD decay rates of the population were found to be 7 to 8 cM and 13 to 15 cM, where the $r^2$ dropped to 0.25 and 0.20, respectively.

### Association mapping of Verticillium resistance

The general linear model (GLM) and mixed linear model (MLM) were used to detect the SSR markers associated with Verticillium wilt resistance among the *G. hirsutum* population. Seventeen loci were found to be associated with Verticillium wilt resistance in GLM at the significant threshold $p \leq 0.01$ after screening the total diversity markers (Table 4). However, only six loci were detected to be associated with Verticillium wilt resistance in MLM when incorporated both population structure and kinship among individuals ($p \leq 0.01$). Some loci such as NAU2265_382, NAU2277_60, BNL1694_415, NAU2741_282, and NAU5099_280, were found to be significantly associated with the traits in both of GLM and MLM. Well, MUSS440_401 was only detected in the MLM, and not was found in the GLM.

Allele effects on the phenotype were calculated (Table 4). Most of the loci showed significant negative effects on the Verticillium wilt resistance. NAU5099_280 appeared the most obvious effect that could reduce 6.22 point of the Verticillium resistance. However, NAU2277_60, BNL1694_415, NAU2741_282 and MUSS440_401 performed the especially abilities of promoting the Verticillium wilt resistance of cotton, which could increase the Verticillium wilt resistance 7.79 and 10.28 respectively ($p \leq 0.01$). The allele effects estimated in this test would be useful for making an accurate selection in the proceeding of molecular marker assistant breeding.

## DISCUSSION

In this study, association mapping was firstly used to identify the genetic markers associated with Verticillium wilt resistance in cotton. The whole genome was scanned with 106 SSR markers that located in the 26 chromosomes. High genetic diversity of population was certified after calculating the PIC and genetic distance of the group. STRUCTURE was used to determine the structure of population, Three subgroups were found with K=3, and then the whole population was assigned to three subgroups and one mixed group separately. The genetic diversity and structure of the sample showed highly effects on the level of LD and the accuracy of the associated loci (Breseghello and Sorrells, 2006). The closely related cultivars in the group would violate the assumptions of the algorithm of Structure (Pritchard, 2000a) and inflate LD among unlinked loci. Thus, high polymorphic cultivars were necessary for the association analysis.

The level of LD was estimated in the population. 11.6%

**Table 4.** SSR markers associated with verticillium resistance among the *G. hirsutum* population.

| Association markers | GLM | MLM | Effect for phenotype |
|---|---|---|---|
| | p-value | p-value | |
| NAU3419_252 | $6.5\times10^{-3*}$ | 0.0757 | -0.89 |
| NAU2265_382 | $6.3\times10^{-4**}$ | $9.5\times10^{-3*}$ | -1.18 |
| NAU2277_60 | $4.4\times10^{-3*}$ | $5.2\times10^{-3*}$ | 1.82 |
| NAU2277_72 | $3.8\times10^{-3*}$ | 0.0652 | -0.96 |
| NAU1190_228 | $9.4\times10^{-3*}$ | 0.1846 | -0.91 |
| NAU2679_218 | $5.1\times10^{-3*}$ | 0.0155 | -1.53 |
| BNL1694_415 | $1.5\times10^{-3*}$ | $6.5\times10^{-3*}$ | 1.15 |
| TMB1963_218 | $5.7\times10^{-3*}$ | 0.0734 | -0.94 |
| TMB1963_243 | $2.7\times10^{-3*}$ | 0.0415 | 1.02 |
| NAU2437_245 | $2.9\times10^{-3*}$ | 0.0244 | -1.17 |
| BNL1694_235 | $2.4\times10^{-3*}$ | 0.0265 | -1.16 |
| NAU1102_230 | $3.0\times10^{-3*}$ | 0.016 | -1.26 |
| NAU3110_224 | $3.5\times10^{-3*}$ | 0.0155 | -1.22 |
| NAU3110_292 | $3.6\times10^{-3*}$ | 0.0174 | -1.22 |
| NAU3110_318 | $3.6\times10^{-3*}$ | 0.0174 | -1.22 |
| NAU2741_282 | $5.3\times10^{-3*}$ | $3.1\times10^{-3*}$ | 7.79 |
| NAU5099_280 | $8.3\times10^{-3*}$ | $8.4\times10^{-3*}$ | -6.22 |
| MUSS440_401 | 0.0101 | $6.4\times10^{-3*}$ | 10.28 |

*, ** indicate significance at the probability levels of p<0.01 and 0.001 respectively.

of loci pairs were found to be linkage disequilibrium. Haplotype LD decayed with the genetic distance of alleles in the sample. The decay rate was about 13 to 15cM (r2<0.2), which was similar to the report of Abdurakhmonov in the landrace stocks germplasm (2009). The decayed distance of LD usually determined the density of markers for the association mapping. Longer decayed distance needed fewer markers to cover the whole genome. In cotton, the total recombination length of genome was 5 200 cM with an average 400 kb per cM (Paterson and Smith, 1999). Our result showed that the LD decay rate of the population was 13 to 15 cM (r2≥0.2), which meant that 300~400 polymorphic loci were required for the association mapping. Though 278 loci detected with 106 markers were fewer than those of theoretical prediction, the number could be reduced to 80~100 if the LD rate was set at the r2≥0.1 threshold with the decayed distance 55 to 65 cM.

The General Linear Model (GLM) and the Mixed Linear Model (MLM) were used to detect the markers of associated with Verticillium wilt. We found that seventeen loci were associated with Verticillium wilt resistance at the significant level of p≤0.01 in GLM. In those associated loci, thirteen loci showed negative allele effects, and four loci showed positive allele effects. When the factor of kinship was incorporated in MLM, most of those loci that were screened in the GLM were filtered, and only six loci were remained to be associated with Verticillium wilt resistance, including four positive effect loci and two negative effect loci. MUSS440_401 locus was only detected in MLM, and not was found in the GLM, which meant that population stratification affected marker–trait association significantly. MLM could filter most of the significant markers detected by GLM, and reduced both false-positive and false-negative rates by adding population structure and kinship as covariate.

The QTLs of Verticillium wilt resistance had been successfully detected through F2 and RIL group based on the method of combination interval mapping in the past years (Wang et al., 2004; Yuksel et al., 2005; Yang et al., 2007, 2008, 2009). Different results were obtained using different linkage group. Four QTLs were detected to be located on chromosome A5, A7 and A8 at the seedling stage, and three QTLs were detected to be located on chromosome A5, A7 and A9 at the mature stage in the combination group of *G. barbadense* × *G. hirsutum* (Yuksel et al., 2005; Yang et al., 2007, 2008). Three QTLs were found to be located on the LG01 linkage group and chromosomes D8 and D7 at the seedling stage, and four QTLs were detected on the LG01 chromosomes A11, D8 and D7 at the mature stage in the group of *G. hirsutum*×*G. hirsutum* (Yang et al., 2007, 2009). Some important markers associated with Verticillium wilt resistance were screened, which were also found to be located on the chromosomes 11, 16, 17, 19, and26 (Zhao et al., 2014). D9 and D7 were found to host most of the

QTLs that were resistant to the *Verticillium* VD8, BP2 and T9 races (Jiang et al., 2009). In present research, all of the loci associated with Verticillium wilt resistance were firstly reported. However, the Verticillium wilt resistance was affected by a complex genetic system including the different *Verticillium* race. We used mixed races to infect the cottons, and calculated the index of resistance after evaluating from the seedling to mature stage. Therefore, the loci associated with Verticillium wilt resistance that we screened were_horizontal resistance, which were useful for MAS in the breeding.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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# Biochemical and secondary metabolites changes under moisture and temperature stress in cassava (*Manihot esculenta* Crantz)

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**Cassava's (*Manihot esculenta* Crantz) importance as a food security crop in Sub Saharan Africa is enhanced by its special traits such as tolerance to drought and high yields under drought stress. However, full understanding of tolerance mechanisms under hydrothermal stress in cassava is a key in developing highly tolerant varieties with increased yield. In our study, the effects of low soil moisture and increased temperature on cassava physiology were investigated. Twenty (20) cassava varieties were evaluated in a Randomized Complete Block Design in western Uganda. Hydrothermal stress was described as a period of no rainfall for a period of eight weeks leading to low soil moisture (contents between 28 to 35%) and average daily temperatures of ≥35°C. The average daily relative humidity during this period was considerably low (≤40%) further complementing already enhanced stress conditions. As such, the contents of important biochemicals and secondary metabolites in the plants were altered in a bid to counteract the effects of stress. Significant differences occurred in accumulation of main biochemicals such as soluble proteins ($P<0.05$), free reducing sugars ($P<0.05$) and bound reducing sugars ($P<0.05$) while reductions in the total starch yield by 70 to 100% of the original composition before stress were observed in all the test varieties. Changes in pigment properties were also observed with a decrease in the total carotenoid content (~65%) and chlorophyll a (*Chla*) (~40%) but no significant changes were observed for chlorophyll b (*Chlb*). Secondary metabolites such as phenolics and tannins too depicted varied but non-significant changes and they existed in low quantities. There were also significant changes in the phenotype (foliar portion of the plant) with at least two mechanisms of tolerance identified. The study showed the importance of carbohydrate and nitrogen cycle related metabolites in mediating tolerance in cassava by affecting their phenotypic expression in the plant.**

**Key words:** Hydrothermal stress, bio-chemicals, pigments, secondary metabolites, cassava.

## INTRODUCTION

Changing and unpredictable climate patterns have resulted into crop losses leading to food insecurity and poverty in a number of African economies. One of the solutions to this changing climate is the use of improved

crop varieties with tolerance to drought and ability to give a decent harvest despite the unexpected changes in climate (Sagoe, 2006). Cassava (*Manihot esculenta* Crantz) is one of such crops being the third most important source of calories in the tropics (FAO, 2010) and depicting various tolerance mechanisms to hydrothermal stresses. Due to its versatile nature, it has been referred to as the "drought, war and famine crop" to much of the countries in sub-Saharan Africa, Asia and South America (Pearce, 2007) where it is already a major staple crop for a number of people (Okgbenin et al., 2010). It therefore has the potential of being considered as part of the solution to improving food security being experienced as a result of changes in rainfall and temperature patterns occurring in various parts of the world (Liu et al., 2008). It can grow on relatively poor soils, is easily propagated, requires little cultivation and can tolerate periodic incidences of drought (Hillocks, 2002). These traits increase its versatility in production and need to further understand their mechanisms so as to develop superior varieties adaptable to the changing conditions.

Alves and Setter (2000) have reported a number of traits and responses including those dependent on morphological, biochemical and physiological behavior that contribute to the resilience of cassava to moisture stress. These induced responses are due to activation of various metabolic pathways resulting into re-establishment of cellular homeostasis as well as structural protection of membranes (Lokko et al., 2007). They are expressed in form of biochemical manifestation like increased enzyme activity and levels of secondary metabolites such as phenolics and tannins, osmotically active solutes such as proline, antioxidant enzymes such as catalase and peroxidase, hormones such as absiscic acid and pigments such as chlorophylls and carotenoids. As a result, the cassava plant is enabled not only to recover after the stress (Okogbenin et al., 2010) but also to regain capacity to restore its normal metabolic activities in a shorter time and produce decent yield.

Owing to its metabolic efficiency under marginal conditions, cassava produces more energy per unit area than other crops under conditions of water stress (El-Sharkawy, 1993). Consequently, the growing of cassava in conditions of minimal rainfall has flourished. It has also been realized that the principal mechanisms that may control tolerance to drought in cassava include its sensitivity and response to changes in atmospheric humidity and soil water status (Fregene and Setter, 2007). Such mechanisms include among others tight regulation of stomatal opening and the ability to retain photosynthetic activity under prolonged water stress. In addition, the crop has a deep root system which enables it to reach water from lower soil layers under extended periods of hydro-stress. Some of the other water conservation mechanisms in cassava include reducing light interception, reducing leaf canopy and size, leaf fall and heliotropic responses (Alves and Setter, 2004). These measures in addition to responses at biochemical level such as increased activity of growth regulators such as absiscic acid and proteins, both regulatory and enzymatic allow cassava to tolerate a range of hydrothermal related stresses.

In as much as the drought tolerance mechanisms in cassava have been partly elucidated, cassava variety specific differences have been observed with a more mixed complex set of mechanisms being expressed in different cassava varieties (Okogbenin et al., 2013). This makes selection for drought tolerant cassava varieties difficult as it would require additional information concerning the levels of tolerance and how these affect the final yield of the plant. Thus, it is important to characterize the various mechanisms displayed by cassava and develop tools that will allow selections to be easily made under certain specific mechanisms. In addition, most of the studies have been focused on understanding the plants reaction to physical stress such as moisture stress (Turyagyenda et al., 2013, Utsumi et al., 2012), moisture and cold stress (Zhang et al., 2010) and stress due to low soil water status (El-Sharkawy, 2007) under controlled conditions that are different from field conditions. There is, therefore, need to characterize the level and type of tolerance under field stress to allow for specific selections in particular environments. In this study, biochemical properties of nitrogen and carbon metabolic pathways and secondary metabolites from the products of these pathways were used to confirm selections for drought tolerant cassava varieties grown under field conditions. The differences in the levels of individual biochemicals and secondary metabolites were used to elucidate traits governing tolerance. These were important in the understanding of the differences in cassava varieties within a certain group that display different phenotypic mechanisms.

## MATERIALS AND METHODS

### Variety selection and establishment of experimental plots

Twenty varieties of cassava were selected based on known parameters of dry matter content, resistance to Cassava Mosaic Disease (CMD) and farmer preference and established in a Randomized Complete Block Design (RCBD) in Kasese, Western Uganda. The trial consisted of two experimental and two control blocks in $81M^2$ plots, with up to 81 plants per plot. The weather and plant response to available conditions were monitored and changes in main metabolites such as free reducing sugars (mainly as glucose), carbohydrates, chlorophylls and secondary metabolites

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varieties at 6-8 weeks post stress (peak stress)

**Plate 1.** Contrasting phenotypes dependent on physiological mechanism for tolerance to drought at 8 weeks post critical stress. **A.** Stay green variety. **B.** Susceptible variety. **C.** Early recovering variety.

such as cyanide, phenolics and tannins were recorded. Weather, soil and water characteristics were also recorded during the trial period using the location specific weather station with capacity for determination of precipitation, moisture and temperature characteristics. This allowed the determination of a critical hydrothermal stress period in which samples were picked on a biweekly basis. To maintain their metabolic state, picked samples were preserved in liquid nitrogen and transported to the laboratory at the National Crops Resources Research Institute in Namulonge, where they were stored at -80°C. The samples were used to study the relationship between metabolites and physiological state of the plant.

### Determination of changes in primary metabolites (carbohydrate and protein contents)

The changes in the yield and level of carbohydrates were assessed by determining starch and reducing sugar contents before and during the hydrothermal stress period. This was meant to determine the effect of hydrothermal stress on the level of stored carbohydrate as the stress time increased. Starch yield was a measure of total extractable starch from the root portion (100 g) as a percentage of total wet mass (Nuwamanya et al., 2011). The starch content was determined after ethanolic extraction of free and bound reducing sugars from 0.1 g starch. Enzymatic hydrolysis of the pure starch (Nuwamanya et al., 2011) and determination of the subsequent released reducing sugars were done (Dubois et al., 1956). Free reducing sugars were taken to be the water extractable sugars at 30°C. Bound reducing sugars were taken to be the hot ethanol extractable sugars at 70°C for 5 min minus the free reducing sugars. Total leaf protein was determined under different experimental conditions using the Bradford assay after extraction using phosphate buffered saline (Hajiboland and Amirazad 2010).

### Determination of pigment concentrations and secondary metabolites

Quantitative measurements for chlorophyll a (*chla),* chlorophyll b *(chlb)* and carotenoids were determined spectrophotometrically by taking their absorbencies at the following wavelengths respectively; 662, 644 and 445 nm (Wettshtein, 1957). Total leaf phenolics were extracted from 1 cm diameter leaf disc cut out of the 5$^{th}$ fully expanded leaf which was placed in 1.4 mL of a methanol:HCl solution (99:1 v/v) and allowed to extract for 48 h at -20°C (Mazza et al., 2000). Absorbance of extracts was read in a spectrophotometer at 725 nm (Dai and Mumper, 2010). The cyanogenic content of the fresh peels and the peeled cassava root was determined after an initial extraction for 3 min of 10 g material in 0.1 M phosphoric acid by hydrolysis followed by reaction with chloramine-T pyridine barbituric acid (Konig Reaction) as developed by Bradbury et al. (1991). The cyanide content of the peelings was also determined in the same way except that the extraction was carried out for 10 min to allow for complete extraction at extended times.

### Data handling and results analysis

The data collected was analyzed using GenStat Discovery Edition (2012). Mean values for each cassava variety/accession were recorded and relationships between different parameters were determined. Microsoft excel software was used to study the trends in the different properties of the plant studied. Trends in the subsequent moisture, rain and related weather data were also determined and related to the observed changes in plant phenol-type. Selections for drought tolerance were confirmed by chloro-phyll content, accumulation of sugars and loss of carbohydrates, accumulation or loss of proteins and ability to recover earlier.

## RESULTS

Based on observed phenotypic characteristics, varieties were ranked according to their response as shown in Plate 1. The groupings included varieties that maintained a moderately high Leaf Area Index (LAI) during hydrothermal stress or stay green varieties (SGV) (Plate 1A). These included varieties such as NASE 2, NASE 3, 0686, MH/0067 and the local variety Magana. However, some varieties completely lost leaves as stress progressed and even the remaining leaves during stress were dechloropyllated and yellowed signifying losses in chlorophyll and related pigments hence little or no capacity to photosynthesize. These varieties showed little or no capacity

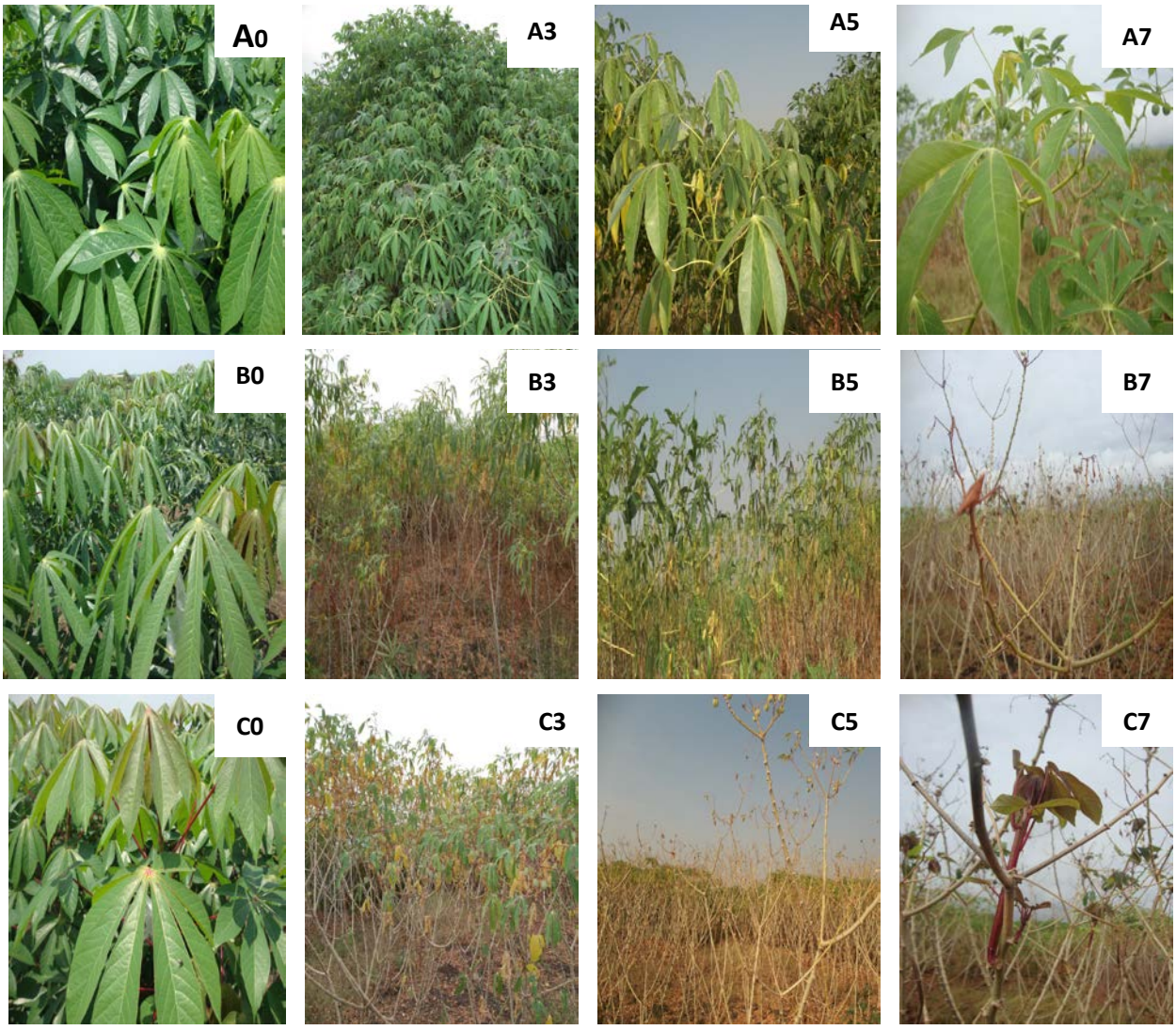


**Plate 2.** Physiological differences in the stay green (A), susceptible (B) and early recovering (C) varieties pre-stress (0), 3 weeks post stress (3), 5 weeks post stress (5) and 7weeks post stress (7).

to recover easily after stress and they were labeled susceptible varieties (SV) (Plate 1B). They included varieties such as NASE 1 and the local variety Rugogoma and Mercury. The other grouping included those that lost all their leaves immediately after onset of stress only to recover immediately with increase in relative humidity or early recovering varieties (ERV) (Plate 1C).

They included NASE 16, NASE 19 and Bukalasa. Some varieties had both mechanisms but were not very pronounced in each case. The variety groupings described were based on phenotypic observations during stress period (Plate 2).

## Defining the critical hydrothermal stress period and selection of tolerant varieties

The results for the average temperature, relative humidity, and related weather conditions during the critical field stress period are presented in Figure 1. The average weather conditions under critical hydrothermal stress were defined by undertaking hourly weather measurements during the stress period. There was no rain during this period with 0 mm of rain received across the two month period. This coincided with reductions in relative humidity by more than 25% with consequent reductions in the dew point up to 8°C. In addition day and night temperatures increased significantly with day temperatures increasing by up to 5°C from an average of 30 to about 35°C while night temperatures increased by 3°C from an average of 18 to 20°C up to 23°C. The increments in temperature resulted into increments in the heat index by about 4 points (Figure 1). Daily average temperatures were high and ranged from 30.7 to 34.9°C during critical stress period compared to the normal averages

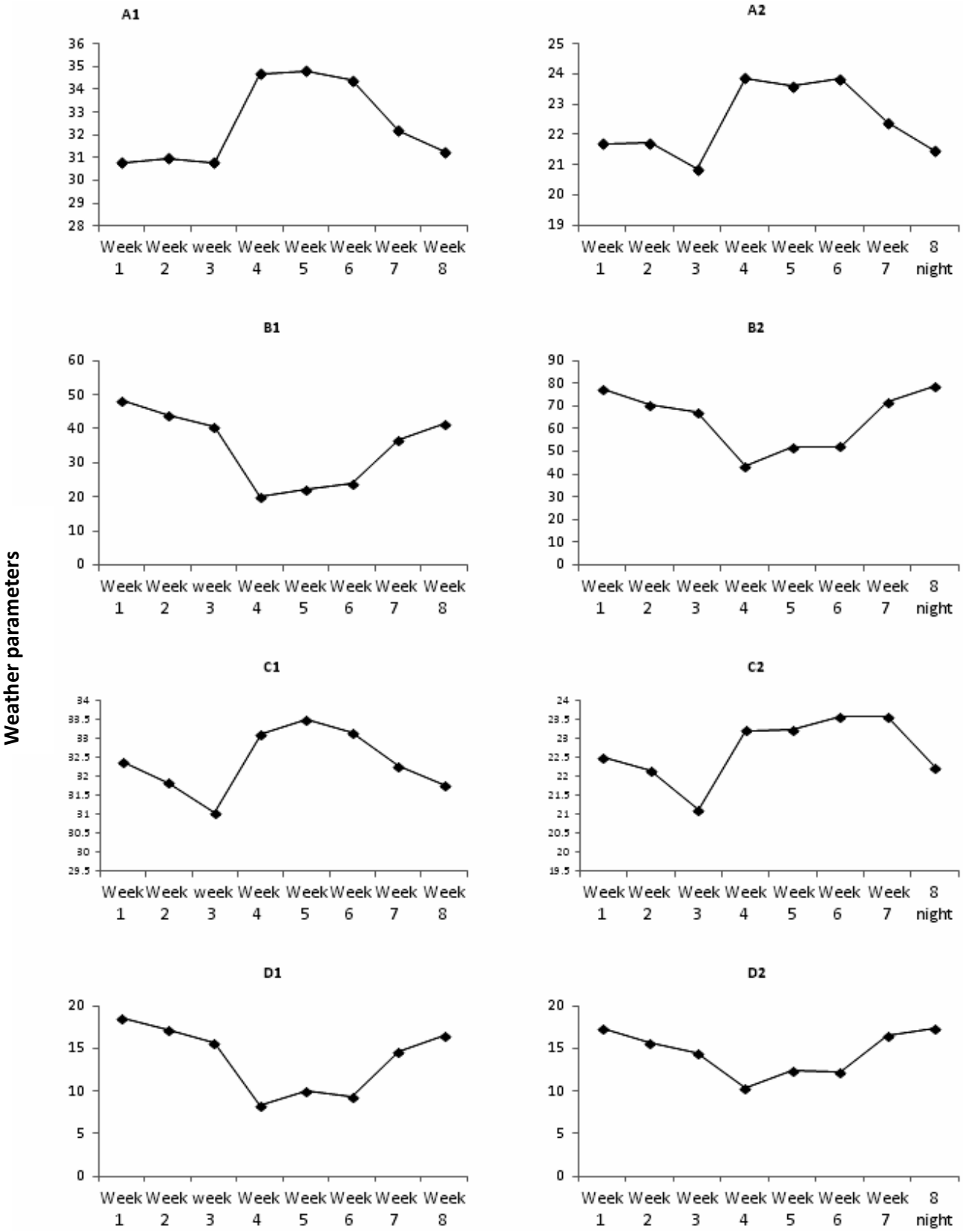


**Figure 1.** Average weekly changes in weather conditions during the critical hydrothermal stress period for both day and night conditions. A1=day temperatures; A2=night temperatures; B1=day relative humidity; B2=Night relative humidity; C1=Day heat index; C2=night heat index; D1=day dew point; D2=night dew point.

of 28.5 to 30.7°C. Week three during critical stress, the temperature increased from 30.5 to 34.9°C and stayed at an average of 34.5°C for three weeks dropping to 31.0°C at the end of the stress period (Figure 1A1). Night temperatures ranged from 20.5 to 23.8°C (Figure 1A2) up from an average of 16 to 18°C during normal conditions. Relative humidity during the day dropped significantly from about 78 to 85% during normal conditions to 22 to 50% in the stress period. The drop followed the same pattern as the temperature (Figure 1B1). At night the relative humidity dropped from 98% during normal conditions down to 48% during the critical stress period (Figure 1 B2).

The heat index described as the felt temperature followed the same pattern as the temperature and was important in making inferences on the effect of heat on the plant. Day heat index increased from 28.5 during normal conditions up to 33.8 during peak stress. At night, the heat index also increased from 18.5 to 23.5 (Figure 1C). High heat indices during the day lead to sun burns on the leaves resulting into increases in the rate of water loss on the plant surfaces. Besides that, heat index may have an effect on leaf aging and general drying of plant fragile organs such as stem tips and young leaves. Day and night dew point also dropped significantly with day dew point dropping from 18 to 22.5°C during normal conditions down to 8.4 to 9.0°C during peak stress period signifying an increase in dry atmospheric conditions and night dew point reducing significantly from 18 to 20°C during normal conditions to about 11 to 13°C during peak stress (Figure 1D). Reduction in mole fraction of water vapor in the atmosphere signified by low dew points indicates an increased evapo-transpiration from plant and soil surfaces. This intensified moisture and heat stress conditions were imposed on the plant during stress.

## Changes in levels of primary metabolites (carbohydrates)

Primary metabolites like carbohydrates and sugars which are products of photosynthesis are easily affected by changes in leaf characteristics brought about by physical changes in the environment. Thus, this study changes in carbohydrate contents were determined in order to understand the efficiency of phenotypic mechanisms observed in selection of drought tolerant varieties and how they relate to stored and metabolisable carbohydrate. These changes are presented in Table 1. Reductions in starch levels were observed with progression of stress time while reducing sugars increased. There was an average decline in starch yield over the stress period in all the varieties although it was well pronounced among the susceptible varieties and the stay green varieties compared to the early recovering varieties (Table 1). The rate of drop in starch yield measured as the gradient was -1.424 for the stay green (Nase 2, NASE 3, I/92/0686 and I/92/00062) and -2.356 for susceptible varieties (Rugogoma and NASE 1) respectively, accounting for approximately 25 to 40% reduction in starch yield. However, in the early recovering varieties, there was a drop of about 25% of the original starch yield after three weeks of the critical stress period with an increase observed by the 5th week of peak stress with an overall positive change of 0.073 accounting for 7.0% increase compared to the original starch yield prior to stress. The differences observed in percentage starch yield in different varieties indicated the use of different mechanisms of tolerance by the different cassava varieties and the dependence of some mechanisms on stored reserves of the plant.

The starch content reduced progressively with time in line with the drop in starch yield observed. A high rate of reduction was observed for stay green varieties (-0.024) compared to susceptible varieties (-0.017) and early recovering varieties (-0.014) for starch content. By the fifth week, the critical stress period of the starch content had reduced to an average of 0.073 g/g of fresh root from an earlier average of 0.15 g/g of fresh root in all varieties although high reductions were observed for stay green varieties. No significant differences ($p>0.05$) were observed for starch content variations across the different varieties, although significant differences were observed within variety groups with the stay green varieties posting higher losses. However, the levels of bound reducing sugars increased with increase in the stress time explaining the possible losses in starch yield and content observed. Reducing sugars were also negatively correlated to starch yield ($r = -0.685$) although they had no significant correlations with starch content. Carbohydrate (starch content and reducing sugar) (Table 1) profile changes revealed an interesting phenomenon in the reaction of cassava plants to stress especially in three broad groups cassava. While starch content was not significantly correlated to either starch yield, free reducing sugars, or bound reducing sugars, it was positively correlated to the cyanide content ($r = 0.269$). Such negative/positive correlation shows that increases in cyanide content do not interfere with stored forms of carbohydrates such as starch though products of carbohydrate metabolism are important in mediating the production of secondary metabolites such as cyanide which are important in plant defense against stress. The starch content was also negatively correlated to the protein content although the correlation was weak ($r = -0.245$). On the other hand, reducing sugars increased with increase in stress and the increments were well pronounced among the stay green, susceptible and early recovering variety groups rather than individual varieties within these groups. There was a general increase in total reducing sugars for all the varieties and variety groups between week one and week three in the critical stress period with the lowest percentage increase observed for the early recovering varieties (12.3%) compared to stay green varieties (44.17%) and susceptible varieties (26.0%). However, initial reducing

**Table 1.** Changes and rate of change (r) in the contents of starch and free reducing sugars over the eight (8) week stress period.

| Group (Δ) | SC0 | FRS0 | BRS0 | SC3 | FRS3 | BRS3 | SC5 | FRS5 | BRS5 | SC7 | FRS7 | BRS7 | rSC | rFRS | rBRS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stay green | 0.133[a] | 0.134[a] | 0.363[a] | 0.177[a] | 0.240[a] | 0.397[a] | 0.075[a] | 0.182[a] | 0.455[a] | 0.077[a] | 0.231[a] | 0.412[a] | -0.023 | 0.023 | 0.110 |
| Early recovering | 0.106[a] | 0.171[b] | 0.348[b] | 0.111[b] | 0.195[b] | 0.787[b] | 0.071[a] | 0.166[a] | 0.276[b] | 0.075[a] | 0.142[b] | 0.278[b] | -0.014 | -0.012 | -0.072 |
| Susceptible | 0.106[a] | 0.168[b] | 0.330[c] | 0.134[c] | 0.227[c] | 0.521[c] | 0.070[a] | 0.170[a] | 0.613[c] | 0.071[a] | 0.239[a] | 0.667[c] | -0.017 | 0.018 | 0.02 |
| LSD | 0.031 | 0.025 | 0.045 | 0.01 | 0.152 | 0.058 | 0.023 | 0.053 | 0.031 | 0.022 | 0.114 | 0.084 | | | |
| P value | 0.461 | 0.031 | 0.018 | 0.018 | 0.068 | 0.0001 | 0.99 | 0.067 | 0.001 | 0.323 | 0.034 | 0.001 | | | |

0, 3, 5, and 7 correspond to values of different carbohydrate metabolites for week 1, 3, 5 and 7 during the critical stress period. SC=starch content; FRS=free reducing sugars; bound reducing sugars; R=rate of change in SC; FRS and BRS during the 8 weeks of stress. Values with different corresponding letters are different at $p<0.05$.

sugar contents were higher for early recovering varieties (0.171 mg/g) compared to stay green varieties (0.134 mg/g) and susceptible varieties (0.168 mg/g). After the fifth week, increases were only observed for stay green varieties (from 0.182 to 0.231 mg/g) and the susceptible varieties (from 0.17 to 0.239 mg/g). In contrast, reducing sugar content values decreased for all the early recovering varieties (from 0.166 to 0.142 mg/g) at the same rate as their increase in other varieties after the fifth week of stress (Table 1).

This is shown by the positive gradient (0.02 and 0.11) for stay green and susceptible varieties, respectively, while a negative gradient was observed for the early recovering at -0.072. Signi-ficant ($p<0.05$) changes were observed for free reducing sugar contents in all test varieties and their quantities were higher in stay green and susceptible varieties compared to early recovering varieties (Table 1). Consistently, free low reducing sugar values were observed across the stress time for early recovering varieties compared to stay green varieties and susceptible varieties up to the end of the stress period and into the recovery phase as shown in Table 1.

Bound reducing sugars had higher contents compared to free reducing sugars in all cases observed and increased with the stress time (Table 1) and ranged from 0.33 to 0.787 mg/g in different varieties at different times of the stress period. Among the stay green varieties, they increased linearly from 0.368 mg/g at the onset of the stress to about 0.46 mg/g by the end of the 5th week post stress. Among the susceptible varieties, the rate of increase in bound reducing sugars was high compared to the stay green varieties with increments from 0.33 mg/g at onset of stress to 0.667 mg/g by the end of the stress period. In contrast, increments in bound reducing sugars among early recovering varieties were observed in the first three weeks after peak stress onset from 0.348 to 0.787 mg/g. After this, bound reducing sugars reduced significantly to 0.274 mg/g by the 5th week post initial peak stress depicting an overall negative gradient throughout the stress period.

## Changes in the fresh root and peel cyanide contents

During stress, cassava plants accumulate nitrogenous compounds such as proteins, cyanide and phenolic substances such as secondary metabolites which are important as defense compounds and mediate vast physiological responses. Results for the cyanogenic potential of the roots and peels through the peak stress period are presented in Table 2. The cyanide content was at least two times higher in the peel compared to the fresh portion of the root with tremendous increments at four weeks post peak stress. Significant variations ($p<0.05$) were observed among the different variety groups with low contents observed among the susceptible varieties (about 168 ppm) pre critical stress period which composed of most of the “sweet” and local varieties. With onset of peak stress, the peel cyanide content was significantly ($p<0.05$) high for early recovering varieties (about 602 ppm) compared to stay green varieties (about 393 ppm) and susceptible varieties (about 386 ppm). In the third week of the critical stress period, specific changes in peel cyanide content were observed for susceptible varieties rising up to about 510 ppm and no significant changes were observed for early recovering and stay green varieties. Furthermore by the 5th week of the critical stress period, increased cyanide levels were observed in the peels with over four times increase in varieties among the groups of susceptible varieties (about 1605 ppm) early recovering (1457 ppm) and stay green (984 ppm) varieties. However by the $7^{th}$ week of the critical stress period, the levels of cyanide in the peel (cortex) had reduced and by the end of the stress period,

**Table 2.** Changes and rate of change(r) in root and peel cyanide content over the stress period.

| Variety group | CR0 | CP0 | CR3 | CP3 | CR5 | CP5 | CR7 | CP7 | CR8 | CP8 | rCR | rCP |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stay green varieties | 0.393$^{a}$ | 0.393$^{a}$ | 0.309$^{a}$ | 0.298$^{a}$ | 0.549$^{a}$ | 0.984$^{a}$ | 0.347$^{a}$ | 0.501$^{a}$ | 0.356$^{a}$ | 0.646$^{a}$ | -0.01 | 0.07 |
| Early recovering varieties | 0.301$^{a}$ | 0.602$^{b}$ | 0.245$^{b}$ | 0.535$^{b}$ | 0.642$^{a}$ | 1.457$^{b}$ | 0.246$^{a}$ | 0.407$^{a}$ | 0.087$^{b}$ | 0.149$^{b}$ | -0.04 | -0.10 |
| Susceptible varieties | 0.168$^{a}$ | 0.386$^{a}$ | 0.381$^{c}$ | 0.510$^{c}$ | 0.405$^{a}$ | 1.605$^{b}$ | 0.301$^{a}$ | 0.667$^{a}$ | 0.070$^{c}$ | 0.286$^{c}$ | -0.03 | -0.01 |
| LSD | 0.177 | 0.241 | 0.032 | 0.036 | 0.283 | 0.082 | 0.09 | 0.192 | 0.214 | 0.315 | | |
| P-Value | 0.242 | 0.03 | 0.0310 | 0.011 | 0.076 | 0.01 | 0.317 | 0.330 | 0.001 | 0.001 | | |

0, 3, 5, and 7and 8 correspond to values of different cyanide contents metabolites for week 1, 3, 5 and 7 during the critical stress period and week 8 during recovery. CR=Cyanide content for the root; CP=cyanide content for the peel; CR=cyanide content for root cortex; R=rate of change in CR and CP during the 8 weeks critical stress period. Values with different corresponding letters are different at $p<0.05$.

the cyanide content had reduced to 149 ppm for the early recovering varieties and 286 ppm for the susceptible varieties but it was still high in stay green varieties at 646 ppm. Root cyanide contents also increased with critical stress time at different rates among the different variety groups. It increased from 393 to 549 ppm among the stay green varieties and from 300 to 642 ppm among the early recovering varieties by the fifth week of the critical stress period. In susceptible varieties, significant increments were observed by the third week from 168 to 510 ppm. Decrease in cyanide content was observed within the recovery phase 5 weeks post initial peak stress dropping specifically in early recovering (from 642 to 87 ppm) and susceptible (from 405 to 70 ppm) varieties. However no significant drops were observed for stay green varieties. Significant ($p<0.05$) differences were observed for root cyanide content among the variety groups at all times during critical stress period. Among variety groups, rate of change in the fresh peeled root was higher for stay green varieties (-0.043) and susceptible varieties (0.028) throughout the stress period. High negative changes in the cyanide content of the peel were observed for the ERVs (Table 2). The relationships between cyanide content and primary metabolites were studied and the results are presented in Figure 2. It was established that increments in cyanide content coincided with increments in protein content and reductions in main metabolites such as starch and sugars. In fact, drops in total carbohydrate content three weeks into the critical stress period coincided with increments in the cyanide content. The same applied to main pigments chlorophyll and carotenoids which also reduced as the protein and cyanide content increased (Figure 2). In the recovery phase, significant drops in cyanide content coincided with a positive gradient for total carbohydrate although the protein content was not affected. This phenomenon was the same for all the varieties although differences were observed in the contents of these metabolites for each variety and within the variety groups.

## Changes in total protein and phenolic compounds

The production of moisture and heat responsive proteins in plants plays a key role in plant tolerance to stress and thus in critical stress times, plants change the amount and number of proteins within stress responsive organs such as leaves (Quietsch et al, 2000). The understanding of the alterations in leaf protein content was thus undertaken and the results for leaf protein contents are presented in Table 3. An increment in total protein content was observed for all the variety groups up to the 5th week of the stress period after which a drop was observed in seventh week of the stress period. There were significant differences ($p<0.05$) among varieties for protein contents at the onset of stress and later within the critical stress period with cumulative increments observed for all varieties between the 3rd and 5th week in the critical stress period. High leaf protein contents were observed in susceptible varieties and ranged from 0.34 to 0.5 mg/g while the least were observed for early recovering varieties and ranged from 0.21 to 0.26 mg/g. Five weeks post stress, the protein concentration was higher in stay green varieties (0.56 mg/g). In the fifth week of stress and at the onset of increments in relative humidity, protein content increased two fold with high increments observed for both stay greens and susceptible varieties and ranged from 0.34 to 0.5 mg/g while the least were observed for early recovering varieties and ranged from 0.21 to 0.26 mg/g. Five weeks post stress, the protein concentration was higher in stay green varieties (0.56 mg/g). In the fifth week of stress and at the onset of increments in relative humidity, protein content increased two fold with high increments observed for both stay

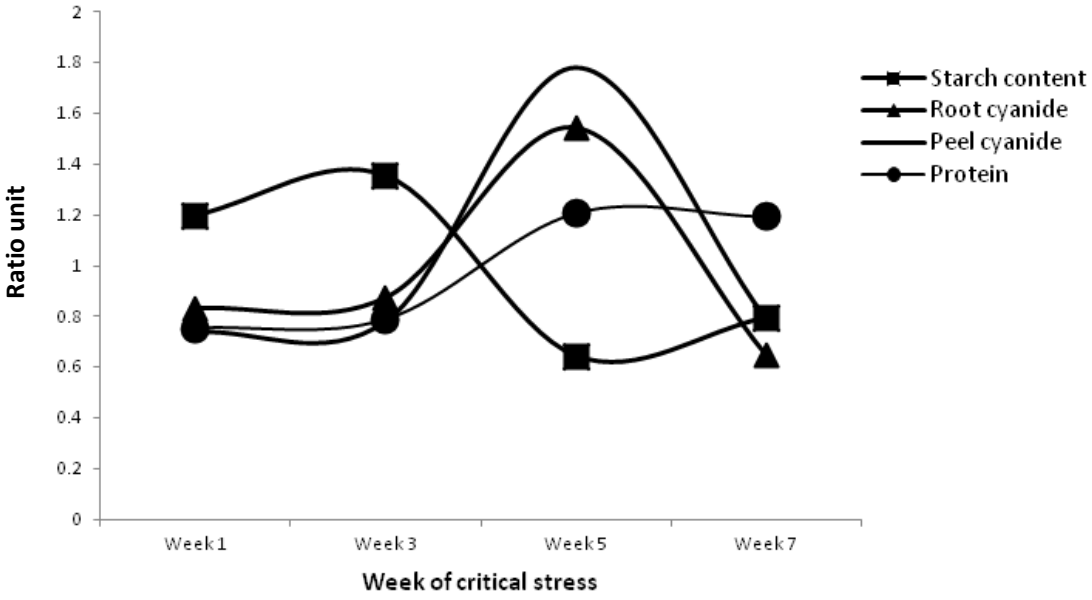


**Figure 2**. Relative changes in main nitrogenous cassava plant metabolites compared to starch content presented as the ratio of concentrations of different metabolites at different times in relation to the final values at the end of the critical stress period.

greens and susceptible varieties. As products of the both carbohydrate and aromatic amino acid path ways, phenolic compounds constitute a range of metabolites such as phenylpropanoids and flavonoids that are important as plant secondary metabolites, defense compounds and as stores of nitrogen. They are affected by changes in plant carbohydrate and amino acid metabolisms and are important indicators of plant response to environmental change. During the critical stress period, phenolic compounds were monitored and the changes in leaf phenolic contents are presented in Table 3. Phenolic compounds concentrations increased at different rates in all the varieties but the increase was more pronounced among susceptible varieties from 0.123 µg/g at onset of stress to about 0.42 µg/g by the 7th week post peak stress. In stay green and early recovering varieties considerable increments were observed five weeks post initial stress with significant differences ($P<0.05$) among the variety groups observed later in the critical stress period. Susceptible varieties accumulated phenolic compounds throughout the stress period even after recovery of other varieties. All varieties had high concentrations of phenolic compounds five weeks post peak stress which reduced with increase in relative humidity during the recovery period except in the susceptible varieties where continuous increments were registered.

## Changes in main plant pigments (chlorophyll and carotenoids) during critical stress

Plant pigments are important in various physiological processes such as photosynthesis in addition to plant defense. Understanding the effect of stress on these pigments allows us to get insights into the plants' mode of resilience to stress and its ability to uphold certain physiological processes such as photosynthesis under optimal stress conditions. Variations observed for leaf pigments mainly chlorophylls and caretonoids across the variety groups and at different times during the peak stress period are presented in Table 4. An overall reduction in the amount of carotenoids was observed during the stress period. Significant differences ($P<0.05$) were observed among variety groups for carotenoid content at the 3rd and 7th week of critical stress. Among the early recovering varieties, carotenoid content remained the same at about 0.345 µg/g at the start of the peak stress period only to increase to about 0.421 µg/g during the recovery phase. In stay green varieties, reductions were observed where carotenoid content reduced from 0.456 µg/g at the start of the peak stress period to 0.356 µg/g by the end of the peak period. No significant increments were observed during the recovery phase for these varieties. In susceptible varieties, the carotenoid contents dropped from 0.511 to 0.378 µg/g by the end of the stress period. Slight increments were observed during the recovery phase for these varieties to about 0.442 µg/g of carotenoids (Table 4). In particular, a drop was observed in total carotenoid content with significant drops in stay green varieties (gradient, -0.041) while in susceptible varieties, a drop was observed between week 1 and 3 post-stress and as well as between week 5 and 7. Minor reductions were observed for carotene content (gradient -0.014) throughout the stress period for susceptible varieties. For early recovering varieties, increments were

**Table 3.** Change in main nitrogenous compound (protein and phenolics) during the eight week critical period.

| Variety group | Protein 1 | Protein 3 | Protein 5 | Protein 7 | Phenolic 1 | Phenolic 3 | Phenolic 5 | Phenolic 7 |
|---|---|---|---|---|---|---|---|---|
| Stay green varieties | 0.027[a] | 0.029a | 0.056a | 0.018a | 0.015a | 0.018ab | 0.019 | 0.015 |
| Early recovering varieties | 0.034[b] | 0.031a | 0.049a | 0.026b | 0.012a | 0.014b | 0.033 | 0.042 |
| Susceptible varieties | 0.021c | 0.026a | 0.022b | 0.018a | 0.015a | 0.026a | 0.012 | 0.014 |
| LSD | 0.003 | 0.01 | 0.0198 | 0.004 | 0.021 | 0.018 | 0.016 | 0.012 |
| P-Value | 0.001 | 0.492 | 0.010 | 0.01 | 0.235 | 0.167 | 0.045 | 0.01 |

1, 3, 5, and 7 correspond to values of different metabolite contents for week 1, 3, 5 and 7 during the critical stress period and week 8 during recovery
Values with different corresponding letters are different at $p<0.05$

**Table 4.** Changes in the pigment content with stress time during the critical stress period.

| Parameter | Chla1 | Chla3 | Chla5 | Chla7 | Chlb1 | Chlb3 | Chlb5 | Chlb7 | Cart1 | Cart3 | Cart5 | Cart7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Stay green varieties | 0.201 | 0.197 | 0.198 | 0.246 | 0.094 | 0.101 | 0.073 | 0.109 | 0.456 | 0.479 | 0.350 | 0.362 |
| Early recovering varieties | 0.122 | 0.152 | 0.218 | 0.252 | 0.061 | 0.068 | 0.086 | 0.106 | 0.335 | 0.377 | 0.373 | 0.421 |
| Susceptible varieties | 0.227 | 0.112 | 0.182 | 0.228 | 0.108 | 0.052 | 0.08 | 0.100 | 0.511 | 0.312 | 0.378 | 0.442 |
| LSD | 0.028 | 0.044 | 0.038 | 0.053 | 0.027 | 0.018 | 0.014 | 0.025 | 0.173 | 0.082 | 0.113 | 0.114 |
| P-Value | 0.001 | 0.02 | 0.009 | 0.004 | 0.001 | 0.01 | 0.151 | 0.015 | 0.153 | 0.029 | 0.755 | 0.034 |

1, 3, 5, and 7 correspond to values of different cyanide contents metabolites for week 1, 3, 5 and 7 during the critical stress period and week 8 during recovery. CR=Cyanide content for the root, CP=Cyanide content for the peel R=Rate of change in CR and CP during the 8 weeks critical stress period.

observed overall (gradient, 0.025) with a slight drop at week 5 and further increments by week 7 at onset of new leaves. A slight drop observed at week 5 coincides with onset of recovery and end of peak stress where only very young leaves were available on most plants.

Chlorophyll a (*chla*) exhibited a different pattern from carotenoids in most variety groups during the stress time. Among the early recovering varieties, *chla* increased from 0.112 µg/g to twice as much as (0.218 µg/g) by the end of the stress period reaching a peak of 0.252 µg/g after recovery and onset of new leaves. However in stay green varieties, *chla* values dropped from 0.204 µg/g at the onset of peak stress to about 0.198 µg/g a rather less significant drop. A major decrease was observed in susceptible varieties where the concentration of *chla* dropped from 0.227 to 0.11 µg/g by the third week post peak stress (Table 4).

Much as *chlb* did not change significantly during stress time, a drop in the concentration of *chlb* was observed in the other variety groups except the early recovery varieties where no specific changes were observed during stress with averages of about 0.068 µg/g only to increase to about 0.106 µg/g during the recovery phase. Among the stay green varieties, concentrations of *chlb* reduced from 0.084 µg at the onset of peak stress to about 0.073 µg/g at the end of stress. The same pattern was observed for susceptible varieties where *chlb* contents dropped by almost half the original value from 0.108 to 0.052 µg/g by the third week of critical stress. At onset of recovery, increments in *chlb* content were observed for both stay green and susceptible varieties (Table 4).

## DISCUSSION

The selection of drought tolerant cassava varieties has been the most elusive and challenging among many cassava breeders. However, the use of combined selection tools both in areas of genomics and biochemistry could provide a lot of insights into possibilities that will help in selection. In particular biochemical tools can be easily determined and used for faster selection. In this study, changes in major biochemical compounds in the

**Table 5.** Correlation analysis for contents of different metabolites mid critical stress period.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *CnPP* | 1 | | | | | | | | | | | |
| *CnPR* | **0.834** | 1 | | | | | | | | | | |
| *RS* | 0.116 | -0.047 | 1 | | | | | | | | | |
| *BRS* | **-0.339** | **-0.418** | **0.518** | 1 | | | | | | | | |
| *Cart* | 0.098 | 0.038 | -0.032 | **-0.496** | 1 | | | | | | | |
| *Chla* | -0.051 | 0.188 | **0.583** | -0.020 | 0.382 | 1 | | | | | | |
| *Chlb* | 0.235 | 0.296 | 0.367 | **-0.442** | **0.659** | **0.752** | 1 | | | | | |
| *FRS* | 0.175 | -0.008 | **0.725** | 0.627 | **-0.346** | 0.084 | -0.001 | 1 | | | | |
| *Phe* | 0.163 | 0.305 | 0.425 | 0.319 | -0.189 | **0.531** | 0.091 | 0.116 | 1 | | | |
| *Prt* | -0.154 | **-0.236** | **0.873** | **0.664** | -0.006 | **0.596** | 0.268 | **0.663** | **0.567** | 1 | | |
| *SCa* | 0.269 | 0.183 | 0.042 | 0.151 | **-0.597** | **-0.308** | **-0.425** | -0.071 | 0.162 | **-0.245** | 1 | |
| *SY* | 0.087 | -0.006 | -**0.685** | -0.097 | -0.219 | **-0.858** | **-0.736** | -0.152 | **-0.432** | **-0.614** | 0.041 | 1 |
| | *CnPP* | *BRS* | *Cart* | *Chla* | *Chlb* | *FRS* | *Phe* | *Prt* | *Sca* | *SY* | *CnPR* | *RS* |

CnPP=Cyanogenic potential for root cortex; CnPR=cyanogenic potential for the fresh root; RS=reducing sugar; BRS=bound reducing sugars; Cart=carotenoid; Chla=chlorophyll; Chlb=chlorophyll b; FRS=Free reducing Sugars; Phe=phenolics content; Prt=protein content; SCa=starch content; SY=starch yield.

main carbohydrate and nitrogen metabolic pathways were investigated and their variations highlighted in different varieties under optimal stress in the field. Changes in carbohydrates were observed during the stress period as their levels decreased with time since they act as a resource for the production of most of the protective proteins and other bio-molecules required in the reversing of effects of stress in plants (Kheder et al., 2003). Loss in total starch yield in all varieties which was observed, is an indicator of the process of remobilization of stored resources in a plant to cater for survival during stress (Setter and Fregene, 2007). In fact, loss in starch was negatively correlated to total reducing sugars (r=-0.685), an indicator that increments in reducing sugars such as glucose where as a result of losses in starch. It was also negatively correlated to *chla* (r=-0.835) and *chlb* (r=-0.736) while it was positively correlated to phenolic content (r=0.432) and the protein content (r=0.614) (Table 5). This shows that losses in starch may be due to shut down of major photosynthetic processes due to loss of photosynthetic pigments but also results into accumulation of important stress metabolites such as proteins and phenolics. In essence, there was reverse translocation of metabolisable sugar resources from the root for leaf and stem growth and maintenance among the stay green varieties accounting for the decline in starch yield as seen in the first three weeks of peak stress (Table 1) have been suggested by (Mir et al., 2012). However, the losses in starch yield differed with susceptible varieties losing more of the starch at any time and faster compared to the stay green and early recovering varieties. For the stay green varieties the remobilized sugars may allow for the maintenance of a decent leaf area index throughout the stress with minimum photosynthesis. This may explain the low drop in sugar contents observed compared to early recovering varieties that had lost all the leaves by 5th week post peak stress. Much as the susceptible varieties maintain a number of leaves, the leaves were usually dechlorophyllated with a low potential for any photosynthetic activity and hence their survival depended on remobilized resources from the root. The early recovering mechanism allowed the plants to shade off all the leaves hence reducing the transpiration rate (Borrell et al., 2000) and the burden of maintaining less photosynthesizing leaves (Tsukaguchi et al., 2003) amid stress and maintaining stored resources. The drop in starch yield in first two weeks explained the coping mechanism by these cassava varieties as they counteracted the effects of the stress. The slight increment in starch yield observed after six weeks coincided with the increase in relative humidity and the reestablishment of young vigorous leaves which started to carry out photosynthesis. The difference in these varieties lies in the fact that while stay greens maintained old but less efficient leaves for photosynthesis, the early recovering varieties easily regain their leaf potential with young vigorous leaves that increased the level of starch deposits in the root immediately after the stress period. Loss of leaves early in stress period by early recovering varieties reflected the ability of these plants to maintain a high level of stored carbohydrate by shutting down most of the metabolic processes carried out by the growing points of the plant during stress. Such plants are better suited for stress management compared to the stay green varieties. Significant losses observed for susceptible and stay green varieties which maintain a high leaf area index points to the fact that the maintenance of a high leaf area index requires more remobilization and hence considerable losses in starch. These varieties are not so well suited for stress management since severe stress will lead to depletion of storage starch from the root hence affecting plant productivity.

Increase in reducing sugar content observed (Table 1) can be attributed to the breakdown of starch and other storage carbohydrates during the stress period. However, the rate of breakdown is an indicator of the level of remobilization of these starches by the different varieties. Sudden increases in reducing sugars content for early recovering varieties (Table 1) is possibly due to the stress shock and the need for more energy for driving a number of processes in the bid to counteract the various stresses. However, the drop in reducing sugars signifies a drop in metabolic activity by these plants conserving both energy and resources in this instance. A linear trend observed for the increase in reducing sugars for the susceptible varieties meant that they do not have any coping mechanisms and hence use their storage reserves to accommodate the effect of the stresses observed at the different time points with increasing stress resulting into continued remobilization. These varieties may also exhibit a high metabolic rate induced by stress that would drain all the available resources putting the plants into a continuous catabolic state.

Meanwhile, the high concentration of bound reducing sugars during peak stress point to an internal genetic mechanism by which plants increase freely metabolizable carbohydrate in order to tolerate various stresses. Consistent increase in bound reducing sugars over the stress time (Table 1) points to continuous remobilization of the starch resources over time as the plant struggles to survive (Mir et al., 2012). In particular, the reduction in the recovery phase may be due to the utilization of the sugars (mainly glucose) for generation of energy that would be used in the anabolic pathways that synthesize plant defensive compounds such as cyanogenic glucosides (Jorgensen et al., 2005) among others. In this study, it was noted that a negative correlation occurred between bound reducing sugars and the cyanide contents for both peels (r=-0.418) and fresh root portion of the plant (r=-0.339) (Table 5). This supports the notion that such secondary metabolites are synthesized using the available sugars. The negative correlation between the cyanogenic potential and protein (r= -0.236) also points to the utilization of amino acids as the nitrogen source in production of cyanide used as a stress management strategy. Although, the differences in the rate of increase of these sugars (Table 1) were differed across variety groups. Such differences may be used in categorizing tolerance in these groups where for instance a high rate of reduction in stay green varieties compared to the early recovering ones was consistent with observed losses in starch yield and described the mechanisms used in these different sets of stay green and early recovering varieties. High remobilizations were required in stay green varieties to maintain the leaf area index and the unshed leaves during stress period. Variations observed in carbohydrate levels also point to the importance of carbohydrate metabolism in mediating stress response. The different metabolic pathways for carbohydrates production and utilization provided the much required precursors for stress responsive metabolites which in turn mediate observed patterns of tolerance or susceptibility. Genes and their products in these pathways therefore could be used to make valid inferences on what happens during carbohydrate metabolism (Fujiki et al., 2000).

Wide variations in the concentration of cyanogens among cultivars of cassava, ranging from 10 to 2,000 ppm hydrogen cyanide equivalent were observed. Similar variations have been observed among cassava varieties growing under moisture and salinity stress where by increased accumulation of cyanide was prevalent (Cardoso et al., 2005). Their accumulation follow a similar trend within the fresh root and the peel with the root accumulating more of the cyanide at any particular time and hence acting as the first line of defense. Cyanogenic glycosides have been shown to play an important role in nitrogen storage in some species (Møller, 2010), which would explain the increments observed away from normal suggestive of the initiation of plants mechanism for nitrogen storage. The drop in cyanide concentration of about 200 ppm observed after stress also points to the importance of nitrogen in growth and development during the recovery phase as suggested by (Lechtenberg, 2011). Moreover, it may also be related to higher yields observed in early recovering varieties as a result of improved nitrogen-use-efficiency and reduced herbivory (Møller, 2010; Lechtenberg, 2011). Significant correlations (r=-0.236) between cyanide and protein content confirmed the increased nitrogen use efficiency in cassava. An increase in cyanide and protein content in relation to the decrease in bound and free sugars (Figure 2) suggests a gene expression mechanism meant to synthesize the relevant stress factors at the expense of carbohydrate metabolism for energy generation. Cyanide accumulation may also suggest roles of increased nitrogen use efficiency by cassava plants during stress which may play important roles during recovery and continued re-growth since it also acts as a sugar storage mechanism (Lechtenberg, 2011). The role of cyanide as the main secondary metabolite in mediating stress tolerance in cassava has been echoed by a number of research initiatives (Yi, 2012; Møller, 2010; Lechtenberg, 2011). Increases in levels of cyanide observed midway the critical stress period followed by the decrease in the amount of free and bound reducing sugars in the 3rd week of stress (Figure 2). This showed that increase in the level of this metabolite allowed the utilization of sugars produced and hence presents a stress management option in the cassava plant (Poulton, 2001). It also indicated an intricately organized pattern where stress recognition mechanisms in the plant allowed for production of free sugars which were then utilized by the plant later in production of stress responsive metabolites including sugars as osmolytes and secondary metabolites such as phenols and tannins (Lechtenberg, 2011;

Yi et al., 2012; Morant et al., 2007). Møller (2010) reported that the role of cyanide in mediating stress was not well known in cassava but it was thought to be a protective agent against other biotic constraints that might have effects on the plant. However, the results from this study suggested that the level of accumulation during stress was important in allowing the selection of better suited varieties for stress resistance. It was also observed that the accumulation of cyanide in different parts of the roots varied among varieties, another factor showing that cyanide accumulation is part of the stress management strategy. Besides, accumulation in the peel appears to be important for protection of the plant against herbivory especially on the root which acts as a storage organ for nutrients during the stress period.

Cassava differentially accumulates proteins in leaves compared to other parts of the plants (Montagnac et al., 2009). Proteins mediate photosynthetic, growth and regulatory functions in the plant with some mainly being enzymes. Plants under severe stress undergo changes which affect the acquisition of nutrients hence affecting the production of storage proteins (Gleadow et al., 2009). However, stress responsive proteins are produced in the plants which occur as secondary metabolites and or osmolytes helping the plant to absorb the limited water from ground (Harding et al., 2003). The enzymes are also important in counteracting the effects of reactive oxygen species observed in many stressed organisms including plants (Apel and Hirt, 2004). The positive correlation between protein contents and pigments such *chla* (r=0.596) also explain their role as protective agents to these important biomolecules. This may explain low protein contents observed at onset of peak stress and their increase with increase in stress time (Table 3) up to a time when the relative humidity had appreciably setting in the recovery phase of the plant. It also coincided with increase in cyanide content (Figure 2) especially for peel from 298 to 984 ppm, an important factor in defending the recovering plant against other biotic stresses like herbivory and pests. Therefore, proteins supplement the role of cyanide as nitrogen storage reserves and secondary defense metabolites for plants during stress helping plants also to go through to the recovery phase using stored resources. The source of these proteins is invariably, the free glucose derived from starch remobilization as shown by the positive correlations between protein and total sugars (r=0.873) and also between protein and bound reducing sugars (r=0.664) (Table 5). Like proteins, phenolic compounds are also important as stress responsive secondary metabolites. They also act as important stores for nitrogen and as a sugar storage mechanism (Wahid et al., 2007). They increase with increments in protein and they were positively correlated to the protein content (r=0.567) and plant pigments (r=0.531) showing that increments in phenolic compounds resulted into increments in pigments. This shows that these compounds act as protective molecules for pigments and other related compounds. Like cyanide, phenolics as nitrogenous secondary metabolite are also important defenses against herbivory, though their specific role in stress tolerance may be as UV screens (Chalker-Scott, 2002) and as osmolytes allowing plants to sustain particular levels of metabolic water (Havaux, 1998). Thus, the role of proteins as secondary metabolites and enzymes and other metabolites such as phenolics during stress in cassava are to maintain a decent nitrogen and carbon source for the plant to use during the period of recovery after stress and also to protect the plant against injury by stress especially for membranes and other important plant structures.

## Conclusion

This study identified main mechanisms of tolerance to drought depending on biochemical characteristics displayed by the plant. These included starch remobilization (reduction of storable carbohydrate resources) and increase in reducing sugar contents (increase in usable carbohydrate metabolites) with consequent negative effects on starch yield. Another mechanism involves the increase in the level of proteins, secondary metabolites like cyanide contents during the stress period and their utilization in form of nitrogen during the recovery phase of the plant. These mechanisms were linked to observed phenotypic differences during stress and where distinct in the three broad phenotypic manifestations observed. Significant relationship between stay green phenotype and *chl* content suggested role playing by *chl* in mediating stress response. The interplay between carbohydrate and nitrogen metabolism in mediating the stay green phenotype was observed. This involved the apparent use of carbohydrate metabolism derived resources in maintaining nitrogen metabolism pathway and its products. In the early recovering varieties, shut down of some if not all elements of carbohydrate metabolism after shedding off of leaves, allowed for reduced metabolism hence more productivity. The results also show that selection for stress tolerance should be done at the right time in the plant growth cycle. Selection for tolerance at a later stage in the plant growth cycle allows the breeders to select for mechanism that maintain a decent yield for the farmer. This also allows the selection of other drought related trait depending on the end user preference especially where the above ground biomass is involved.

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# *Agrobacterium* mediated genetic transformation of popular Indica rice Ratna (IET 1411)

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**This is the first report of genetic transformation of indica rice cultivar Ratna (IET 1411). *Agrobacterium tumefaciens* strain LBA 4404 carrying a binary vector pCAMBIA1301 harboring the CaMV35S promoter driven modified GUS gene was used. Various parameters critical to rice transformation were optimized including callus induction medium, bacterial concentration, co-cultivation conditions, concentration of the plant growth regulator 2,4-D and the concentration of acetosyringone. The transformed lines were analyzed using PCR for marker sequence and gusA gene expression by GUS activity assays. The regeneration and transformation frequency were calculated to be 70 and 47% respectively. This is a reproducible high efficiency transformation technique for the Indica rice cultivar Ratna (IET 1411).**

**Key words:** Indica rice, *Agrobacterium tumefaciens,* callus induction, transformation, regeneration, GUS.

## INTRODUCTION

Rice (*Oryza sativa* L.), the staple primary food crop for more than one third of the world's population, has emerged as a model crop for genome and proteome analysis (Goff, 2002). It serves more than two third of the world population and more than 90% of the Asian population (Khush and Brar, 2001), particularly in tropical and sub-tropical regions. The population of the world is increasing at the rate of 1.17% per year (World Bank, World Development Indicators). The unceasingly expanding population and decrease in arable land area have caused difficulty in meeting people's need. In the same light, the consumption of rice is increasing every year at the rate of 1.8%. But the production of rice has slowed. It is estimated that rice production has to increase by 50% by 2025 (Khush and Virk, 2000) to be enough to feed the people. In order to solve this problem,

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**Abbreviations: MS,** Murashige and Skoog, 2,4-D, 2,4-dichlorophenoxyacetic acid, **CH,** casein hydrolysate.

development of rice varieties with higher yields, excellent grain quality and resistance to biotic/abiotic stresses is the only option. The production of high yielding disease resistant varieties through breeding has reached a plateau and the only viable option left is through transgenic approach (Duan et al., 2012).

The first genetic transformation of rice was carried out by using poly ethylene glycol (PEG) (Uchimiya et al., 1986; Zhang and Wu, 1988; Datta et al., 1990). Subsequently, gene transfer through electroporation and micro projectile bombardment became popular (Christou and Ford, 1995). Various genetic systems have been employed for transformation of rice *via* particle gun delivery (Ghosh et al., 2013). However, *Agrobacterium* mediated transformation of rice is still the method of choice because of its higher transformation efficiency, ability to transfer large fragments of DNA, minimal re-arrangement of the host DNA, and low cost. *Agrobacterium* mediated transformation is a superior method also because it results in stable and low copy number integration of foreign genes into the plant chromosomes (Sahoo et al., 2011).

Rice cultivated in Asia has been classified into two groups: Indica *(Oryza sativa* subsp *indica)* and *japonica (Oryza sativa* subsp *japonica),* of which *indica* rice is more popular (Agrama et al., 2010). But compared to Japonica most of the Indica rice cultivars are recalcitrant and less responsive in tissue culture as well as to *Agrobacterium* mediated transformation. Till date *Agrobacterium* mediated transformation protocols are available for only a few *indica* rice cultivars. Previous reports of Indica rice transformation include the cultivars IR64, IR72 (Kumar et al., 2005), Pusa Basmati1, Swarna (Ramesh et al., 2004) Chaitanya, Phalguna (Ramesh et al., 2004), DS20, OMCS97, ADT 39 (Tyagi et al., 2007). Saika and Toki (2010) reported a new protocol for transformation of *indica* rice cultivar Kasalath while Sahoo et al. (2011) standardized *Agrobacterium* mediated transformation of IR64. In spite of all these efforts, rice being recalcitrant and non-uniform in response to *Agrobacterium* mediated gene transfer, high efficiency protocols for *Agrobacterium*-mediated transformation is still not available for most of the Indica rice cultivars including cultivar Ratna (IET 1411).

Ratna (IET 1411) is a very popular elite Indica rice cultivar of TKM-6 × IR-8 parentage with dwarf size, long slender grains, grain yield of 45 to 50 ton $ha^{-1}$ with a cultivation time of 130 to 135 days, grown vastly throughout India and Southeast Asia. The major problem for its cultivation is that it is susceptible to most of the rice insects, bacterial and fungal diseases, resulting in the high yield loss every year. To overcome this problem, transgenesis is the most obvious solution by which multiple traits like insect /fungal /bacterial resistance genes can be incorporated. But at present there is no available protocol for *Agrobacterium* mediated transformation for Ratna.

Here we present the first transformation protocol for Ratna. We have standardized the critical parameters of transformation, like callus induction prior to infection by *Agrobacterium*, co-cultivation period with *Agrobacterium*, concentrations of Acetosyringone in co-cultivation medium, callus induction medium, Hygromycin concentration and concentration of plant growth regulator 2,4-D. This protocol gave us high regeneration and transformation efficiencies. The transformed Ratna rice lines have been tested by PCR for hygromycin phosphotransferase gene sequence and GUS assay. The regeneration and transformation frequency are 70 and 47% respectively.

## MATERIALS AND METHODS

### Plant material

Seeds of *indica* rice cultivar Ratna (IET1411) were obtained from Rice Research station, Chinsurah, West Bengal, India.

### Surface sterilization of rice seeds and callus induction

Dehusked rice seeds are surface sterilized with 30% (v/v) sodium hypochlorite with 0.01% (v/v) of Tween 20 (Merck, Germany) for 30 min. Seeds were rinsed thoroughly with sterilized distilled water five times. Surface sterilized seeds are transferred onto callus induction medium (CIM) that is MS medium (Murashige and Skoog, 1962) supplemented with 2,4-Dichlorophenoxy acetic acid concentrations of 2 mg $L^{-1}$, 2.5 mg $L^{-1}$ or 3 mg $L^{-1}$ . Different concentrations of casein hydrolysates were used, pH was adjusted to 5.2-5.6, then finally incubated at 28°C in the dark. Within 7 days, callus development was observed from the scutellum tissue. After calli grew to a size 4 to 5 mm in diameter, they were separated from the seeds and transferred to the fresh media for further proliferation. The calli were sub cultured every 10 days interval up to 2 months before infection with *Agrobacterium*.

### *Agrobacterium* strain and binary vector

*A. tumefaciens* strain LBA4404 harboring pCAMBIA 1301 binary vector was used for rice transformation. The T-DNA construct of the vector contains hygromycin phospho transferase gene (*hpt)* as the plant selection marker. It also contains *GUS* as a reporter gene under CaMV35S promoter and *nos* terminater. Figure 1 shows the T-DNA region of the vector. The vector confers kanamycin resistance as bacterial selectable marker under the control of CaMV35S promoter.

### *Agrobacterium* culture, infection, co-cultivation and callus selection

*A. tumefaciens* strain LBA4404 harboring pCAMBIA 1301 was maintained on Luria Bertani (LB) agar (1%) medium. A single colony of bacteria was inoculated in 50 ml of liquid AB medium (Hiei et al., 1994) containing 50 µg $ml^{-1}$ kanamycin and 50 µg $ml^{-1}$ rifampicin and allowed to grow for two days at 28°C on an orbital shaker at 160 rpm. The culture was centrifuged at 4 000 rpm for 10 min and resuspended in AAM media (Hiei et al., 1994). $OD_{600}$ was adjusted to 0.9 to 1.0. The proliferating rice calli were transferred to fresh media and cultured for 3 to 5 days before the *Agrobacterium*

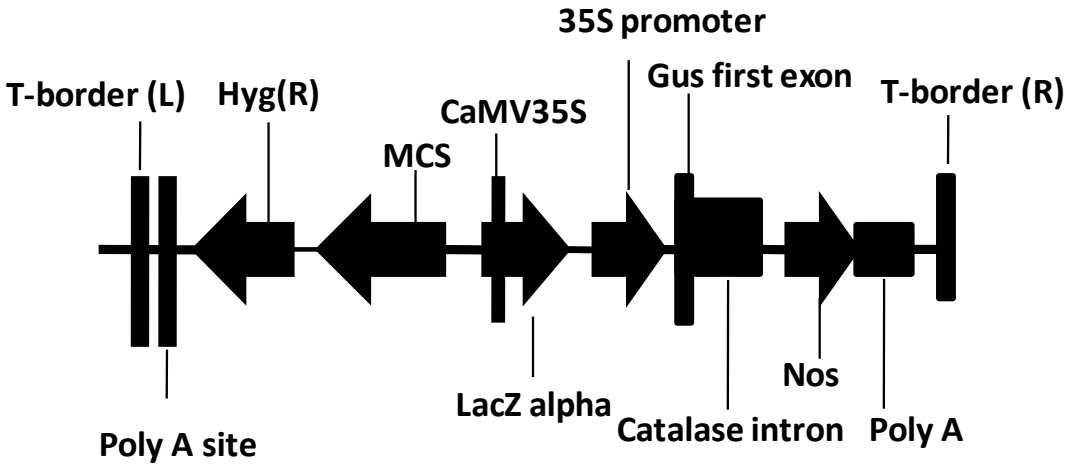


**Figure 1.** Partial map of binary vector pCAMBIA 1301. T-DNA region containing intron-*gus* and *hpt* plant expression cassettes.

infection. The calli were immersed in the bacterial suspension for 10 min. Excess bacteria were soaked on sterilized filter paper (Whatman no.1).

For standardization of co-cultivation, the inoculated calli were transferred either directly onto the co-cultivation media (CCM) or placed on a sterilized filter paper soaked in 1 ml of AAM media, the paper being placed on co-cultivation media (CCM) in 9 cm diameter Petri plates (Rashid et al., 1996). Co-cultivation was carried out in the dark at 25°C for 3 days. Two different concentrations (100 and 200 µg $ml^{-1}$) of acetosyringone (Sigma-Aldrich) were used in this experiment in AAM as well as in the CCM.

After 3 days of co-cultivation under 25°C, infected calli were washed three times in sterilized distilled water followed by a final rinse in 250 µg $ml^{-1}$ cefotaxime solution. Calli were dried on sterilized filter paper and transferred onto selection medium 1 (SM-1). After 15 to 20 days on the selection medium, proliferating portions of the calli were excised with sterile scalpel and transferred onto SM-2 and incubated for 15 days before these were transferred onto SM-3 media.

#### Plantlet regeneration

The proliferating nodular cream-white calli from SM-3 are transferred onto the regeneration medium MMS with or without 20 µg/ml Hygromycin and 250 µg $ml^{-1}$ cefotaxime and incubated at 28±1°C under 16/8 h light/dark photoperiod. After 4 to 5 weeks of incubation, shoot development started from putative transformed calli.

#### Genomic DNA isolation

Genomic DNA was isolated from the callus and leaf tissue using CTAB method with slight modifications (Saghai et al., 1984). 200 mg of leaf tissue was crushed with 1ml CTAB buffer in a sterile mortar. 1 ml of this slurry was taken in a microfuge tube and kept in a water bath set at 65°C for 30 min with occasional mixing. The slurry was centrifuged at 12 000 rpm for 10 min and the supernatant was collected. Equal volume of chloroform: isoamyl alcohol (24:1) was mixed thoroughly with the supernatant by vigorous shaking and centrifuged at 12 000 rpm for 10 min. The upper aqueous layer was collected and 0.8 volume of isopropyl alcohol was mixed with it. Following 10 min of incubation in ice, the mixture was centrifuged at 12 000 rpm for 10 min. The pellet of genomic DNA thus obtained was washed with nuclease free 70% ethanol, dried and dissolved in 50 µL of nuclease free deionised water.

#### PCR analysis for presence of hygromycin phosphotransferase gene

PCR was done to confirm the integration of the T-DNA into the genomic DNA of rice. The forward primers for hpt sequence were 5'GCTCCTACAAATGCCATCA3' and the reverse primer used was 5'GATAGTGGGATTGTGCGTCA3'. PCR reaction cycles was carried out as follows- initial denaturation for 5 min at 94°C, 35 cycles of 30s at 94°C, 45s at 55°C and 1min at 72°C; final extension at 72°C for 10 min. PCR products were visualized in 1% agarose gel stained with 0.5 µg $ml^{-1}$ ethidium bromide.

#### Gus activity assay

The expression of GUS was assayed in rice calli essentially as described by Rueb et al. (1994) with 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc) as substrate. Segments of rice calli were incubated in phosphate buffer (50 mM $NaPO_4$, pH 6.8) at 37°C for 1h. The buffer was decanted and fresh phosphate buffer containing 1.0 mM X-Gluc and 20% methanol was added to the calli. The mixture was vacuum infiltrated into the tissues for 5 min and then the tissues were incubated in that mixture at 37°C overnight. Finally, the tissues were visually examined under stereo microscope (Radical, India). The GUS positive calli showed dark blue patches.

## RESULTS

### Overview of *Agrobacterium* mediated transformation of Ratna (IET 1411) rice

Healthy mature dehusked IET 1411 rice seeds were surface sterilized with 30% aqueous solution of sodium hypochlorite for 30 min, washed with sterile distilled water and placed on MS medium supplemented with 0.5 $gL^{-1}$ CH and 4 $mgL^{-1}$ of 2, 4-D for callus induction. In this method, we have used a simple callus induction medium

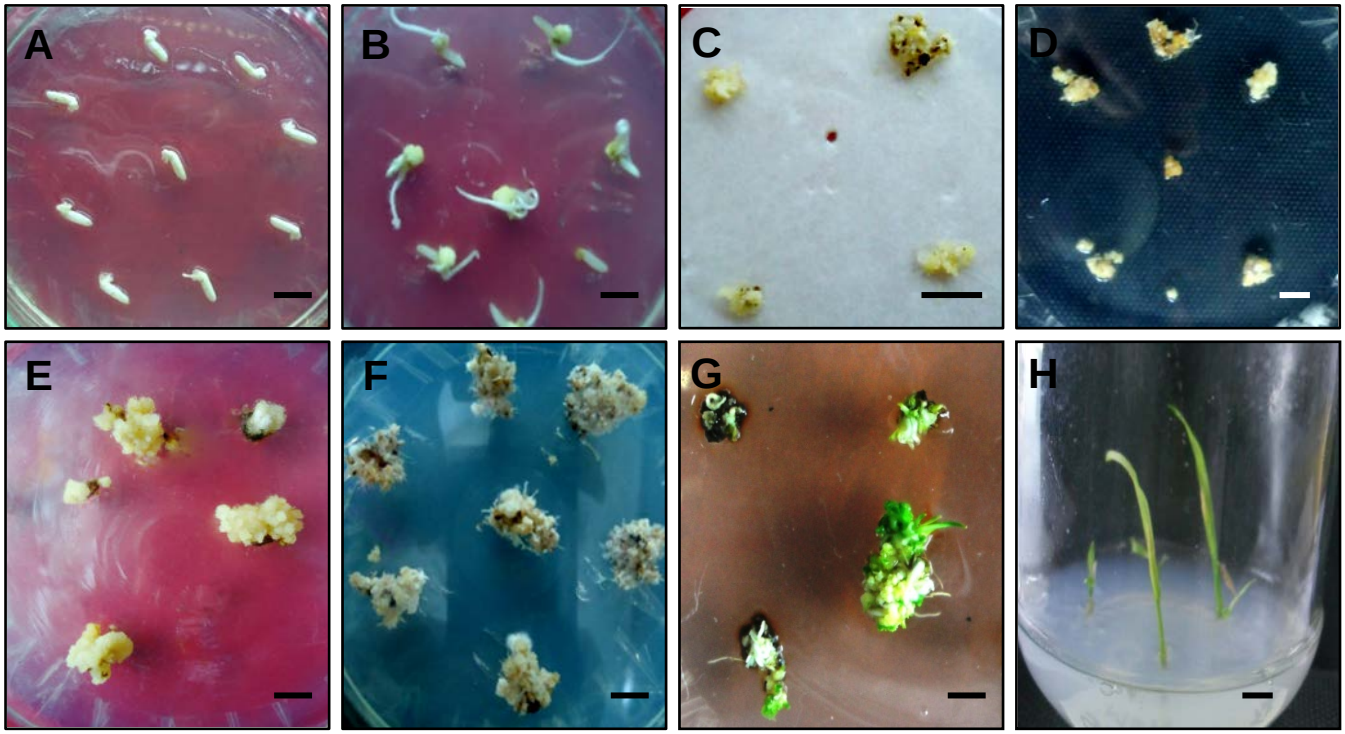


**Figure 2.** Steps for *Agrobacterium* mediated transformation and regeneration from rice calli. (A) Sterilized seed explants; **(B)** callus initiation on callus induction medium (CIM). **(C)** co-cultivation of calli with *Agrobacterium tumefaciens* (LBA4404). **(D, E, F)** Putatively transformed calli on first, second and third selection media that is SIM1, SIM2, SIM3 respectively. (G) Shoots regenerated from transformed calli on regeneration media. (H) Transformed shoots of rice on ½ strength MS agar medium for initiation of roots. Steps A-C was followed in the dark. Bars represents 3 mm.

with only 2, 4-D and without the use of any other hormones or plant growth regulators. These seed explants were sub-cultured onto fresh callus induction media (CIM) with the same composition every 10 days for 2 months. Non-viable brown parts of the calli were removed with scalpel during each transfer. Figure 2A shows the initial stage of callus induction from rice seed explants 2 days after placement on media and Figure 2B shows 10 day old calli. Actively proliferating parts of the calli were used subsequently for infection with *Agrobacterium*.

For preparation of bacterial culture, *A. tumefaciens* strain LBA4404 harboring pCAMBIA 1301 binary vector was cultured in liquid AB media supplemented with 50 µg $ml^{-1}$ Kanamycin. Rice calli were infected using *Agrobacterium* culture with $OD_{600}$ value of 0.35-0.66 resuspended in AAM media. This optimized concentration of *Agrobacterium* is much lower than previous protocols for other idica rice varieties. The inoculated calli were transferred on a sterile filter paper soaked in AAM media which was placed on co-cultivation media (CCM) containing 100 µg $mL^{-1}$ acetosyringone (Figure 2C). Co-cultivation was carried out in the dark at 25°C for 3 days. Infected calli were washed with cefotaxime and transferred to the selection medium SM-1 (Figure 2D). After 15 to 20 day of incubation the proliferating calli were transferred onto SM-2 (Figure 2E) and subsequently onto SM-3 (Figure 2F). The viable calli were transferred onto the regeneration medium MMS with 20 µg $ml^{-1}$ hygromycin and 250 µg $ml^{-1}$ cefotaxime and incubated at 28±1°C under 16/8h light/dark photoperiod (Figure 2G). To induce root initiation, putatively transformed regenerated shoots were separated from the callus tissues, placed in MS basal media of half the strength with 1% agar, pH adjusted to 5.8 for rooting (Figure 2H). The whole work flow for the *Agrobacterium* mediated transformation of IET 1411 rice is summarized in Figure 3.

## Standardization of callus inducing medium

Callus induction as well as regeneration potential of tissue is affected not only by type of explants but also the by the culture conditions and composition of the culture medium including concentration of plant growth regulators (Revathi and Pillai, 2011). It has been reported that the particular genotype of explants used was an important factor for successful embryogenic callus induction and regeneration (Rueb et al., 1994).

MS-CH media was used as callus induction medium along with the best suited hormone supplement that is, 4 $mgL^{-1}$ 2-4D (Figure 4A to L). For standardization of callus induction, four different media compositions were used as

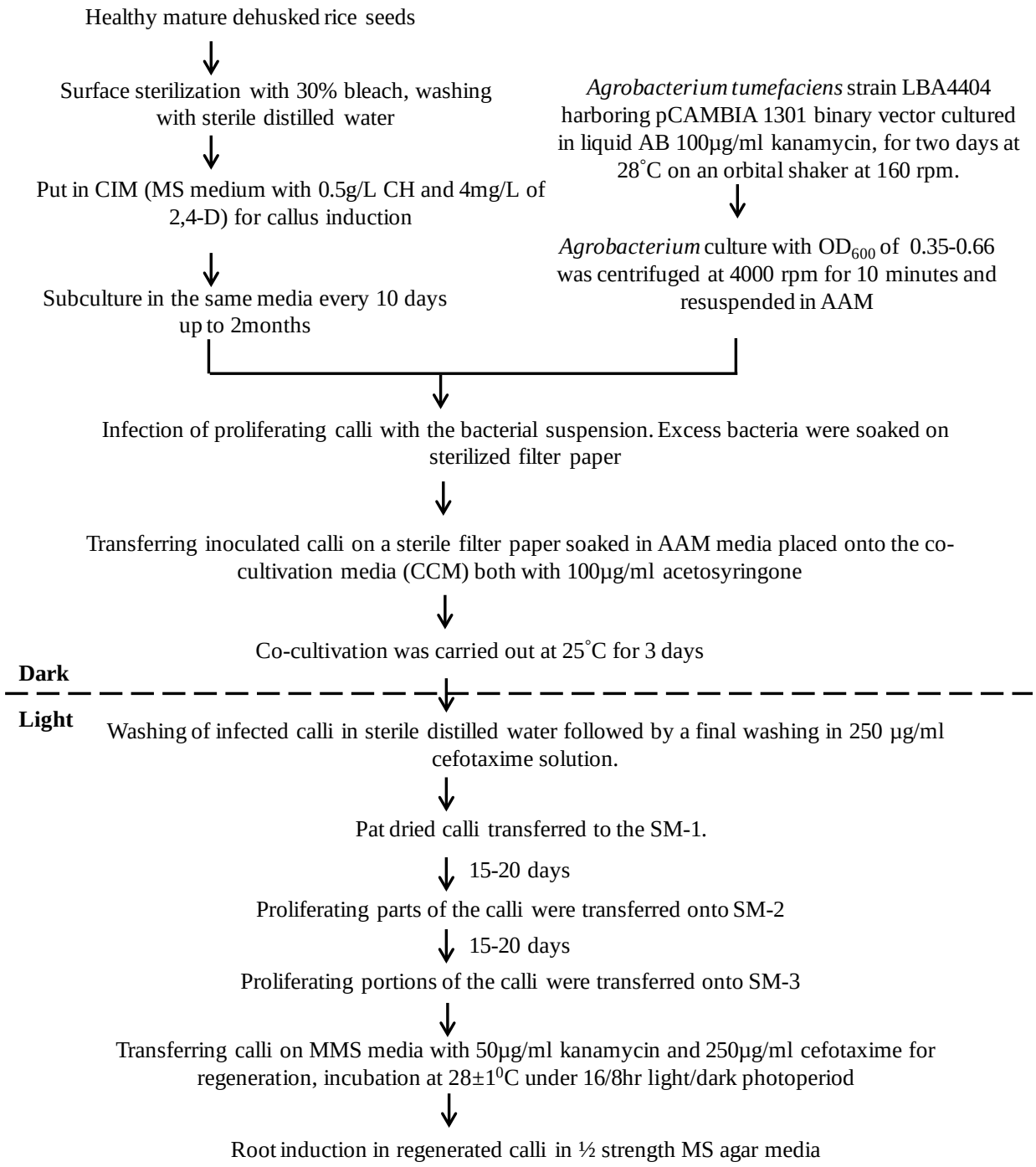


**Figure 3.** Schematic workflow of the protocol**.** The flow chart briefly describes the steps of *Agrobacterium* mediated transformation of IET 1411 rice Ratna.

shown in Table 2. In the case of CIM2 where proline was added, lower percentage of callusing of about 72% was obtained with respect to CIM1. This is in accordance with earlier reports (Rahman et al., 2011). Here the best callus induction frequency was about 85% with CIM1 (Figure 5C). The average callus size was larger of about 6 mm in diameter.

The presence of proline or maltose or the absence of CH reduced the frequency of callusing. After placement of the seeds on this media, callus development started within 3 to 4 days from the scutellum tissue. This primary callus developed for 5 to 10 days growing to 2 to 6 mm in diameter. Approximately, 85% seeds developed mature embryogenic callus from primary callus. This mature

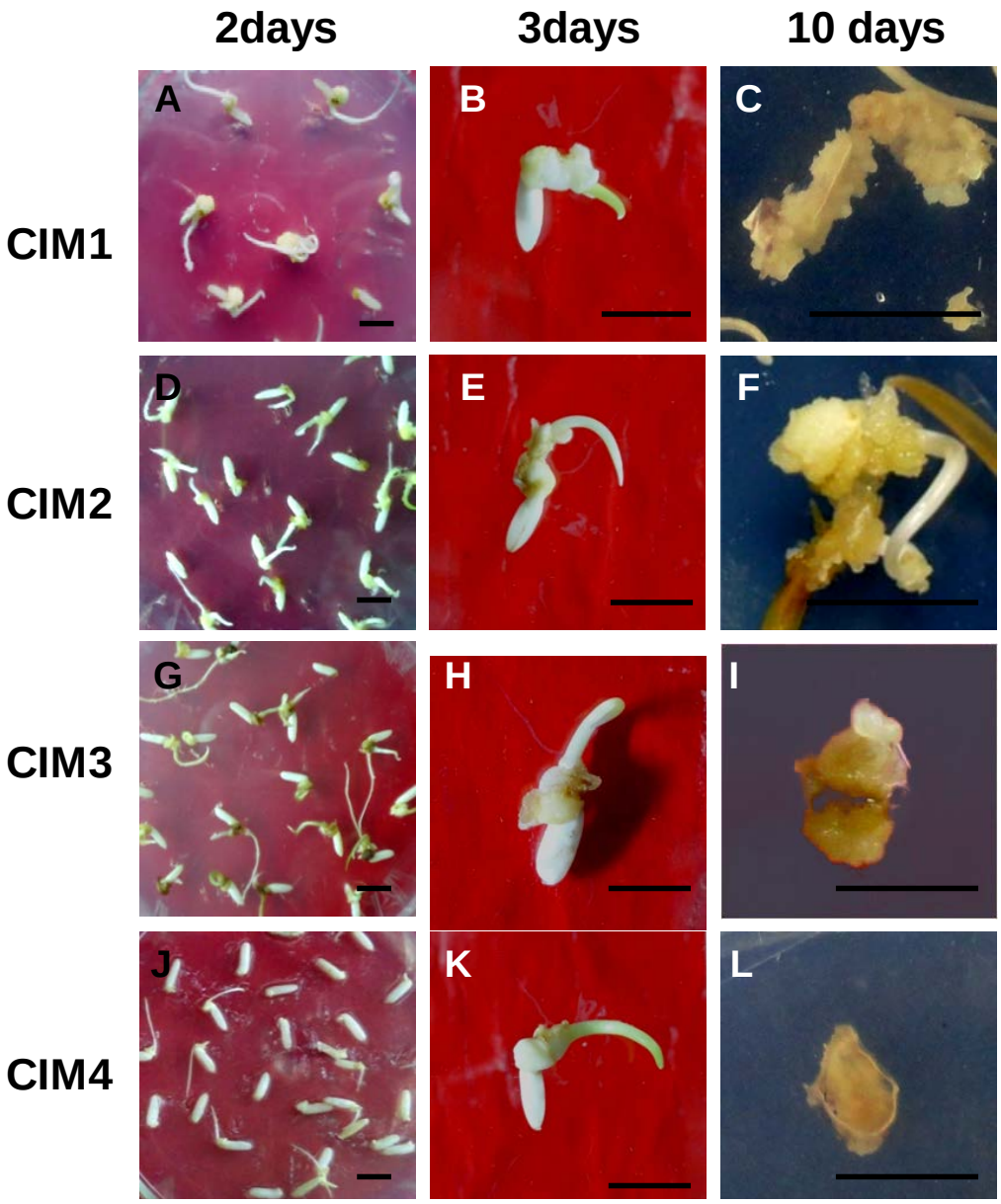


**Figure 4.** Morphological differences between rice calli in different callus inducing media. Left column shows multiple explants at 2 days after inoculation. Middle and right panel shows morphology of an individual callus at 3 days and 10 days after inoculation in callus inducing media; A, B, C, CIM1; D, E, F, CIM2; G, H, I, CIM3; J, K, L, CIM4. Bars represent 3 mm.

**Table 2.** List of different callus inducing media (CIM) used.

| Media | Composition |
|---|---|
| CIM 1 | MS-CH, 2-4D 2.5 mg $L^{-1}$, pH~5.8. |
| CIM 2 | MS-CH, Proline (2.5 g $L^{-1}$), pH~5.8. |
| CIM 3 | MS, Maltose (30 g $L^{-1}$), 2-4D 2.5 mg $L^{-1}$, pH~5.8. |
| CIM 4 | MS, 2-4 D 2.5 mg $L^{-1}$. |

embryogenic, nodular, creamish white, friable calli were maintained for 5 days before infection with *Agrobacterium.*

2, 4-D is known to facilitate callusing from rice seed explants. Different concentrations of 2,4-D were used for optimization. Development of calli from seeds were more frequent on the callus inducing media containing 4 $mgL^{-1}$ 2,4-D in comparison to those containing 2.5 and 3 $mgL^{-1}$ 2,4-D (Figure 5C). Moreover these calli were proliferative and bigger in size. The colour of these compact calli was creamish white. But further increase in the concentrations of 2,4-D influenced negatively on the compactness and colour of calli. Therefore 2, 4-D at 4 $mgL^{-1}$ concentration was found to be most suitable and induced the highest frequency (87.7±4.6%) of callusing from the explants

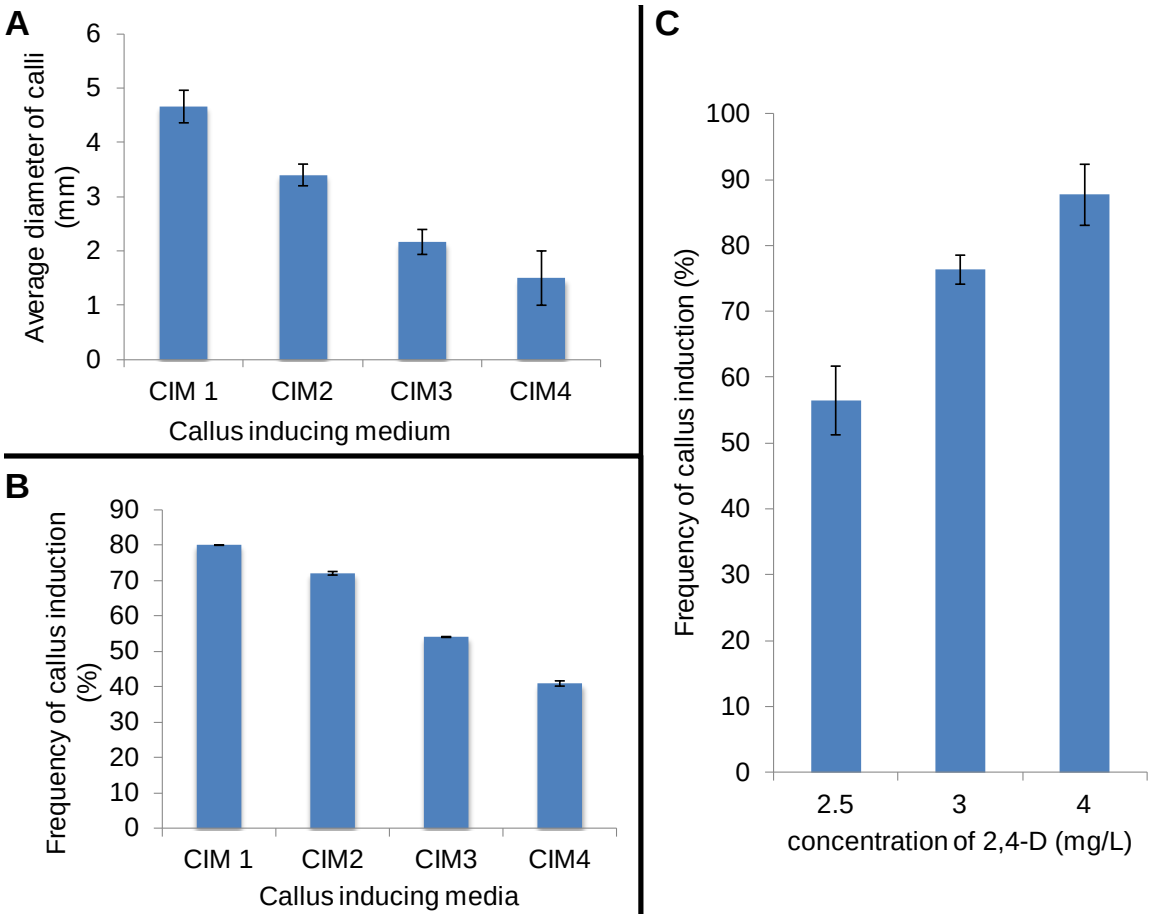


**Figure 5.** Optimization of media components for callus induction. Effect of different media compositions (CIM1, CIM2 CIM3 and CIM4) on (A) size of calli and (B) frequency of callus induction. (C) Effect of concentration of 2,4-D on frequency of callus induction.

(Figure 5C). Further increase in the concentration of 2, 4-D resulted in decrease and non-uniformity in the frequency of callusing (data not shown). The intermediate concentrations of 2.5 and 3 $mgL^{-1}$ induced 56.47±5.2 and 76.3±2.2% callusing (Figure 5C). Hence, subsequent experiments were done with 4 $mgL^{-1}$ 2, 4-D.

## Regeneration of transformed rice plantlets

Mature embryogenic calli were used to standardize the regeneration medium for this variety of rice. Older calli more than 7 days became non-embryogenic. Such calli went on proliferating, when subcultured on regeneration medium but failed to form plantlets. In our study 5 days old calli were most suitable for regeneration.

Regeneration was successful in the modified MS media (MMS) containing MS salts, $B_5$ vitamins, CH 2 $gL^{-1}$, maltose 30 $gL^{-1}$, sorbitol 30 $gL^{-1}$, BAP 4 $mgL^{-1}$, NAA 0.2 $mgL^{-1}$. After placement of the calli on regeneration media and incubated at 28±1°C, 70% of the calli regenerated small shoots within 2 to 3 weeks (Figure 2G).

## Effect of concentration of *Agrobacterium* for inoculation of calli, co-cultivation condition and co-cultivation period on transformation efficiency

Prior to infection, the *Agrobacterium* was cultured in liquid AB medium containing inorganic salts and glucose as carbon source to increase its virulence (Hiei et al., 1994). An experiment was designed using seven different densities of *Agrobacterium* cells, $OD_{600}$ being 0.1, 0.13, 0.15, 0.20, 0.38, 0.66 and 0.69 (Rahman et al., 2011). Different durations of co-cultivation periods (2, 3 and 5 days) were also used to optimize the conditions. Calli cocultivated for 2 days showed negligible GUS expression (0.6 to 1% of calli) and excess period that is, 5 days of cocultivation resulted into damage and browning of calli. Hence, 3 days of cocultivation period was optimum for regeneration from transformed calli. GUS expression was observed and its percentage was quantified as a measure of transformation frequency. The result shows that with 0.66 optical density of *Agrobacterium* culture, the transformation efficiency was maximized (Figure 6A to H). During *Agrobacterium* infection, OD600 of the bacterial

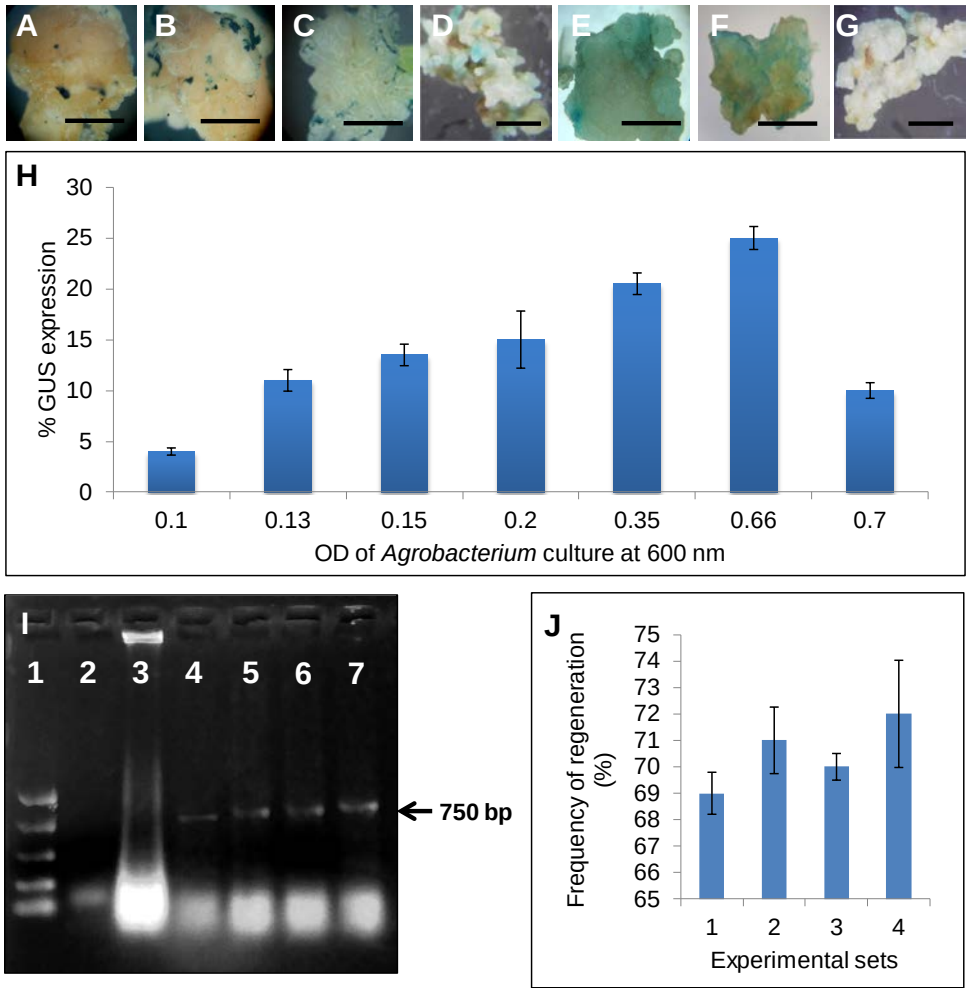


**Figure 6.** Effect of *Agrobacterium* concentrations on transformation efficiency of rice calli and analysis of transformed rice plants. GUS expression in individual calli transformed with *Agrobacterium* cultures having $OD_{600}$ = 0.1 (A), 0.13 (B), 0.15 (C), 0.2 (D), 0.38 (E), 0.66 (F), 0.69 (G). Bars =2mm (A-G). (H) Percentage of GUS expression in calli transformed using different concentration of *Agrobacterium*. Each bar represents mean from three experiments ± standard deviation. (I) PCR analysis of DNA isolated from transformed rice plantlets. Lane 1, DNA marker; lane 2, PCR of genomic DNA from untransformed rice plantlet as negative control; lane 3, positive control; genomic DNA of transformed rice tissue; lanes 4 to 7; PCR products from genomic DNA of transgenic rice calli carrying hpt gene (750 bp) (Line 1-4). (J) Regeneration frequencies in transformed rice of different experimental sets.

suspension must be adjusted within 1.0 because high concentrations of bacteria caused serious injury of the callus thus lowering the transforming efficiency. Co-cultivation with filter paper (Ozawa, 2009) increased transformation frequency because the filter paper prevented overgrowth of bacteria. In the co-cultivation media we used cysteine which reduces browning of the calli (Ozawa, 2009), aceto-syringone and glucose to induce the *vir* gene activity.

## Selections of hygromycin resistant calli

After *Agrobacterium* infection, infected calli were selected on the callus induction media with hygromycin as selection agent. Hygromycin clearly demarcated the transformed calli from non-transformed tissues which failed to regenerate. After first round of selection, only proliferating portions of the calli were transferred onto the fresh selection medium for second round of selection and so on (Figure 2E to G).

## GUS positive shoots indicated correct integration and expression of the T-DNA

Competence was assured by having an established totipotency while expression of gusA gene immediately confirmed that these calli were also competent for transformation. High frequency of GUS expression was

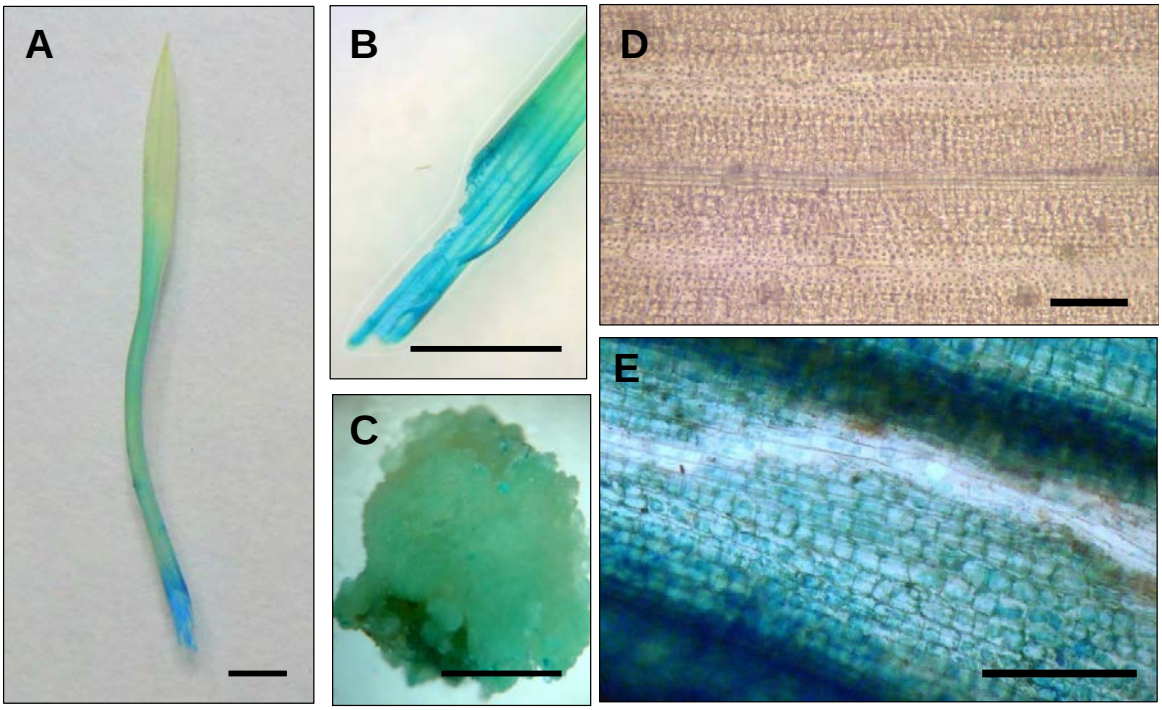


**Figure 7.** Histochemical assay for expression of the *gus*A gene in calli and mature leaf tissues of *Agrobacterium*-transformed rice. (A) Gus expression in transformed rice leaf. **(B)** a leaf sheath. (C) transformed callus of rice. **(D)** Foliar histology of untransformed control. **(E)** Transformed rice plant after GUS histochemical assay. Bars in A, B and C represent 3mm and bars in D, E represent 50 µm.

routinely observed between 4 to 25% (Figure 6H). The expression of GUS reporter gene was found to be a reliable indicator for subsequent production of stable transgenic lines. The shoots that developed from transformed GUS positive rice calli were also GUS positive. Uniform blue colouration in the leaf tissues of transformed plantlets signified the stable integration and expression of the T-DNA in the plant genome (Figure 7A, B). Figure 7D, E shows clearly difference between the untransformed control and the GUS positive leaf cells of rice. Figure 7C shows GUS expression in a transformed callus.

Regenerated shoots that developed from the hygromycin resistant calli, were all PCR positive for presence of *hpt* gene sequence. This data indicate that T-DNA was properly integrated into the genomic DNA of these plantlets. GUS assay was performed for all the hygromycin resistant calli as well as the first leaves of regenerated shoots. All the calli and the leaves showed blue coloration when incubated in the X-gluc solution indicated expression of the GUS transgene.

### Confirmation of presence of hygromycin phosphotrasferase transgene in rice calli by PCR

In the present study hpt gene was used as the selectable marker and hygromycin B as the selective agent for screening of transformed calli. The transformation efficiency of this experiment was measured by the proportion of *hpt* positive calli to the total number of rice calli produced. Genomic DNA of transformed calli was analysed by PCR using specific primers for hpt gene. PCR analysis confirmed the integration of a fragment of expected size 750 bp in the transformed lines (Figure 6I). Out of 22 calli of rice *hpt* gene was integrated in 11 PCR positive calli. Hence the transformation frequency was 47%.

## DISCUSSION

Rice transformation systems have relied on the use of protoplast, microprojectile bombardment and *A. tumefaciens* (Cheng et al., 1998; Repellin et al., 2001; Panahi et al., 2004; Vila et al., 2005; Zaidi et al., 2006; Ramesh et al., 2009). Immature embryos were the first explants to be used for successful transformation of rice back in 1993 which resulted in embryogenic calli (Li et al., 1993). Subsequently many attempts resulted in successful transformation of Japonica rice. On the other hand, transformation protocols for *Agrobacterium* mediated transformation Indica varieties are few. The transformation efficiency of the indica rice variety using *Agrobacterium* is much lower than that of japonica cultivars and leaves room for improvement (Tie et al., 2012). Paucity of reports of successful development of transgenic indica rice indicates the difficulties in the tissue culture and transformation of indica. There are multiple

causes behind the sensitivity of indica rice to tissue culture systems and its poor response in transformation (Ge et al., 2006; Zaidi et al., 2006). The main reason being the resistance of Indica rice to infection by *Agrobacterium* which has been directly linked to defense related gene expression (Tie et al., 2012). Manipulation of plant growth regulators as well as organic and inorganic components and salts within the culture media was done in previous reports for establishment of a media suitable for callus induction of indica rice (Ge et al., 2006).

In this study, a reproducible transformation protocol was standardized using seed explants of indica rice cultivar Ratna (IET 1411). For induction of callus, we have optimized a media with minimum number of components. In this callusing medium, 2,4-D was used as the only growth regulator which induced callusing in 87.7% explants. In previous reports a number of growth regulators including Phenyl acetic acid (NAA, BAP, PAA), kinetin in different combinations with 2,4-D have been used but with less efficiency (Tiwari et al., 2012; Ge et al., 2006). In various systems of rice tissue culture supplements like proline, piclorum, maltose etc. were an inevitable part of the callus inducing media. The present protocol includes a new a callus inducing media (CIM 1) with casein hydrolysate as a supplement and 2,4-D. Though addition of either proline or maltose in the MS basal media showed callus induction as well as proliferation of the calli, the maximum callus induction was observed in CIM 1 without these components.

The modified transient GUS expression assay (Rahman et al., 2011) proved to be a rapid method for standardization of concentration of the *A. tumefaciens* cell suspension and optimization of the transformation efficiency. The optimum $OD_{600}$ of *Agrobacterium* cell suspension deduced with this protocol partially supports the result of other reports of efficient *Agrobacterium* mediated transformation system (Sahoo et al., 2012). Though previous studies (Rahman et al., 2011) considered the *Agrobacterium* cell suspension with $OD_{600}$ value 0.8 to 1 as optimum for rice transformation, here the transformation efficiency obtained during transient GUS expression assay gradually increased with increase in $OD_{600}$ value of bacterial cell suspension up to 0.66 but higher concentration of bacterial probably resulted in damage of explant tissues.

Several factors influence the successful transfer of T-DNA into rice of which Acetosyringone, being the induction agent of *vir* genes, is a crucial one. Earlier research used 150 µM Acetosyringone for transforming indica rice and obtained the maximum transformation frequency of 12% and 200 µM Acetosyringone decreased the percentage of regeneration (Sahoo et al., 2012). The regeneration frequency of Ratna (IET 1411) was 4 times higher when 100 µM acetosyringone was used. Higher concentration of Acetosyringone (200 µM) in cocultivation media resulted in lower frequency of regeneration likely due to necrotic effect of acetosyringone (Sreeramanan et al., 2009).

As the Indica rice Ratna (IET 1411) used in the present report is regularly grown in rice producing fields of India and other parts of south East Asia, this protocol will be useful in transgenesis using superior traits. Moreover it seems likely that the transformation system established here would help in transgenic development of closely related genotypes.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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*Full Length Research Paper*

# Fine structural observations on oocyte development, oogenesis and fertilization process of *Cotugnia polycantha* Diamar, 1893 (Cestoda:Davaineidae) infecting doves (*Streptopelia senegalensis*)

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**Adult *Cotugnia polycantha* was recovered from the intestine of infected *Streptopelia senegalensis.* The fine structure of the ovary, oogenesis and fertilization process are described. The oocytes undergo characteristic cytological changes, that lead to oocyte maturation. Early stages of mature oocytes showed synaptonemal complexes in the nucleus. Lipid droplets appeared in late stages of mature oocyte. Mature oocytes are filled with clusters of cortical granules adjacent to the oocyte plasma membrane. Syncytial interstitial tissue and a number of myelin-like bodies are observed in the ovarian cytoplasm and they fill the cytoplasmic spaces. Oocapt is well-developed, with closely packed musculature beneath the epithelial layer. Wall of the distal oviduct (fertilization canal) bears cilia and lamellae. The fertilization process of *C. polycantha* and different stages of a fertilized oocyte showing vitellocytes with heterogeneous shell-globule clusters are recorded for the first time.**

**Key words:** Ultrastructure, oogenesis, davaineidae, ovary, *cotugnia polycantha*, *streptopelia senegalensis*.

## INTRODUCTION

True tapeworms are exclusively hermaphrodite; they have both male and female reproductive organs in their bodies. The reproductive system includes one or many testes (male reproductive organ), and a single lobed or unlobed ovary with the connecting oviduct and uterus (female reproductive organs) (Cheng, 1986; McDougald 2003). In most tapeworms species, the male organs mature first. The female reproductive system of Platyhelminthes shows great morphological variability with significant differences in the anatomical organization and cell structure by taxonomy (Adiyodia and Adiyodi, 1988). Light microscopical studies on tapeworm oogenesis and embryogenesis were presented by Douglas (1963) and Rybicka (1967). A detailed ultrastructural studies of the ovary and oogenesis have been carried out for a few groups of parasitic Eucestodes (Davies and Roberts, 1983; Poddubnaya et al., 2005a,b and 2007 Poddubnaya et al., 2010; Taeleb and

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Abdel-Moaty, 2011). Several TEM studies have been published on the ultrastructure and differentiation of vitellocytes in cestodes (Świderski and Xylander, 2000). Vitellocytes in cestodes have two important functions: to form eggshell and nourish early embryo (Świderski and Xylander, 1998, 2000). During cestode evolution, these two functions are intensified or much reduced in different taxa, depending on the type of the embryonic development, degree of ovoviviparity and life cycles (Świderski and Mokhtar, 1974; Świderski and Mackiewicz, 1976; Świderski and Xylander, 2000; Świderski et al., 2004a, b).

The genus, *Cotugnia* was created by Diamare 1893 with its type species, *C. digonopora* Pasquale 1890, which was collected from the domestic fowl, *Gallus domesticus* from Africa, India, Burma, Indonesia, Phillipines. Since then, 36 species have been reported to belong to *Cotugina*. *Cotugnia* is the sole representative of the family Davaineidae from birds (Karmveer and Jaywant, 2011).

The aim of this present study is to describe in details the ultra structural ovarian tissue of *C. polycantha* and its oogenesis, giving particular attention to the relationship between the vitellocytes and the fertilization process as well as the formation of fertilized oocytes.

## MATERIALS AND METHDODS

Adult *C. polycantha* was recovered from the intestine of infected *S. senegalensis* collected from Aborawash area, Giza Governorate, Egypt. The worms were processed for morphological examination and described. For ultra structure examination, mature segments of worms collected were cut into small pieces and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 4 h at 4°C; they were rinsed in 0.1 M sodium cacodylate buffer (pH 4.7) at 4°C and then post fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 h at 4°C. Fixed specimens were washed in 0.1 M sodium cacodylate buffer (pH 7.4) , dehydrated in a graded series of acetone and embedded in a mixture of Araldite and Epon. Semithin sections were cut using LKB ultramicrotone, stained with methylene blue and examined using a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined using an AMT camera / Philips 208 transmission electron microscope (TEM).

## RESULTS

### Ovary

The mature proglottides of *C. polycantha* were formed by two sets of male and female genitalia.The ovary is lobulated and located in the lateral portion of the proglottides.

### Ovarian epithelium

Each ovarian follicle is enveloped by a compressed epithelial sheath lying on the basal matrix, that shows epithelial projections into the lumen of the ovary. The epithelial nuclei are located close to the base (basal part) of the ovarian follicle.

The nuclei contain patches of peripheral and central heterochromatin, and the epithelial cytoplasm around every nucleus is filled with ribosomes and isolated mitochondria [Figure 1 (numbers 1, 2)]. Every follicle contains numerous oocytes at different stages of maturation and its size increases continously [Figures 1, 2 and 3 (numbers 3, 6, 8, 10)]. The oocytes undergo characteristic cytological changes, which lead to different stages of oocyte maturation. The electron density of the cytoplasm of some oocytes is much greater than that of others. This probably indicates the higher numbers of ribosomes within the cytoplasm [Figures 2 and 3 (numbers 6, 8,10)].

Oocytes are loosely packed within the follicles and are often hexagonal or oval in outline [Figures 2 and 3 (numbers 4, 8,10)]. Their plasma membrane is generally smooth [Figures 2 and 3 (numbers 6, 8, 9, 10)].

### Oogonia

The oogonia is often spherical and characterized by a relatively small nuclear-cytoplasmic ratio and by a large nucleus containing dense chromatin patches. It is located in the peripheral regions of the follicles. Its electron-dense cytoplasm is filled with ribosomes and few mitochondria. A centriole is present within the cytoplasm [Figures 1 and 2 (numbers 4, 5)].

### Primary oocytes

The early stages of the oocytes are located in close vicinity of the follicular epithelium. These cells are relatively small (12 x 9 µ) and have a large nucleus with prominent nucleolus. Their cytoplasm is packed with free ribosomes and contains mitochondria arranged peripherally. During the growth of the oocytes, the number of mitochondria and volume of the cytoplasm increase. Granular endoplasmic reticulum and golgi complexes structures are observed. [Figures 2 and 3 (numbers 6, 7, 8)].

### Secondary oocytes

Secondary oocytes are larger in size with a smaller nucleus [Figures 3 and 4 (numbers 8-14)]. Mitochondria, granular endoplasmic reticulum and golgi complexes are well developed; they are located throughout the cytoplasm [Figures 3 and 4 (numbers 8,13-15)]. A centriole is located between newly formed nuclei, [Figure 4 (number 12)]. The most striking feature of these cells is the appearance of vitelline materials [Figures 3 and 4 (9, 11, 12, 14)] and homogeneous electron-dense granules arising from the golgi complexes within the cytoplasm

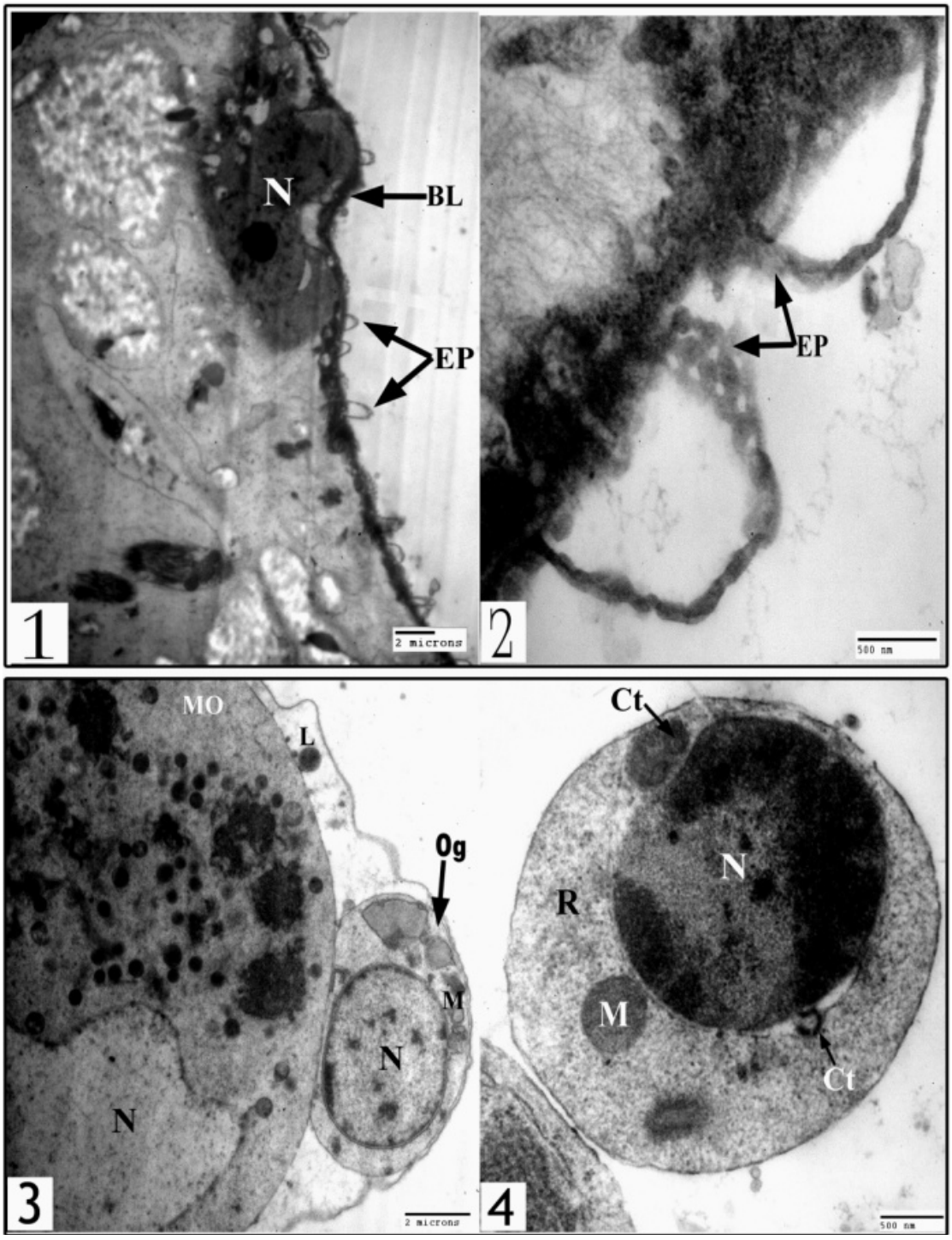


**Figure 1.** Transmission electron micrographs of the ovarian tissue and oocyte development of *Cotugnia polycantha*. **(1)** Ovarian epithelial wall lying on basal layer, showing epithelial projections into the lumen of the ovary Scale bar = 2 microns. **(2)** Lager magnification of the compressed epithelial layer with narrow projections. Sacle bar = 50 nm. **(3)** Early oogonia located adjacent to mature oocyte within interstitial cytoplasm , that contains interstitial lipid droplet . Note cortical granules and vitelline fragments are located peripherally in the mature oocyte. Scale bar = 2 microns **(4)** Oogonia with nucleus contiaing patches of heterochromatin. Mitochondria, ribosomes and pair of centeriole are located withIn the cytoplasm. Scale bar = 500 nm. BL, basal layer; CG, cortical granules; Ct, centeriole; EP, epithelial projection; FO, fertilized oocyte; GC, Golgi complex; GER, granular endoplamic reticulum; GV, Golgi vesicles; L, lipid; M, mitochondria; MO, mature oocyte; M**v**, microvilli; N, nucleus; n, nucleoules; Og, oogonia; PO, primary oocyte; R, ribosomes; SO, secondary oocyte; SG, shell globules; Sp, spermatozoa; VM, vitelline material.

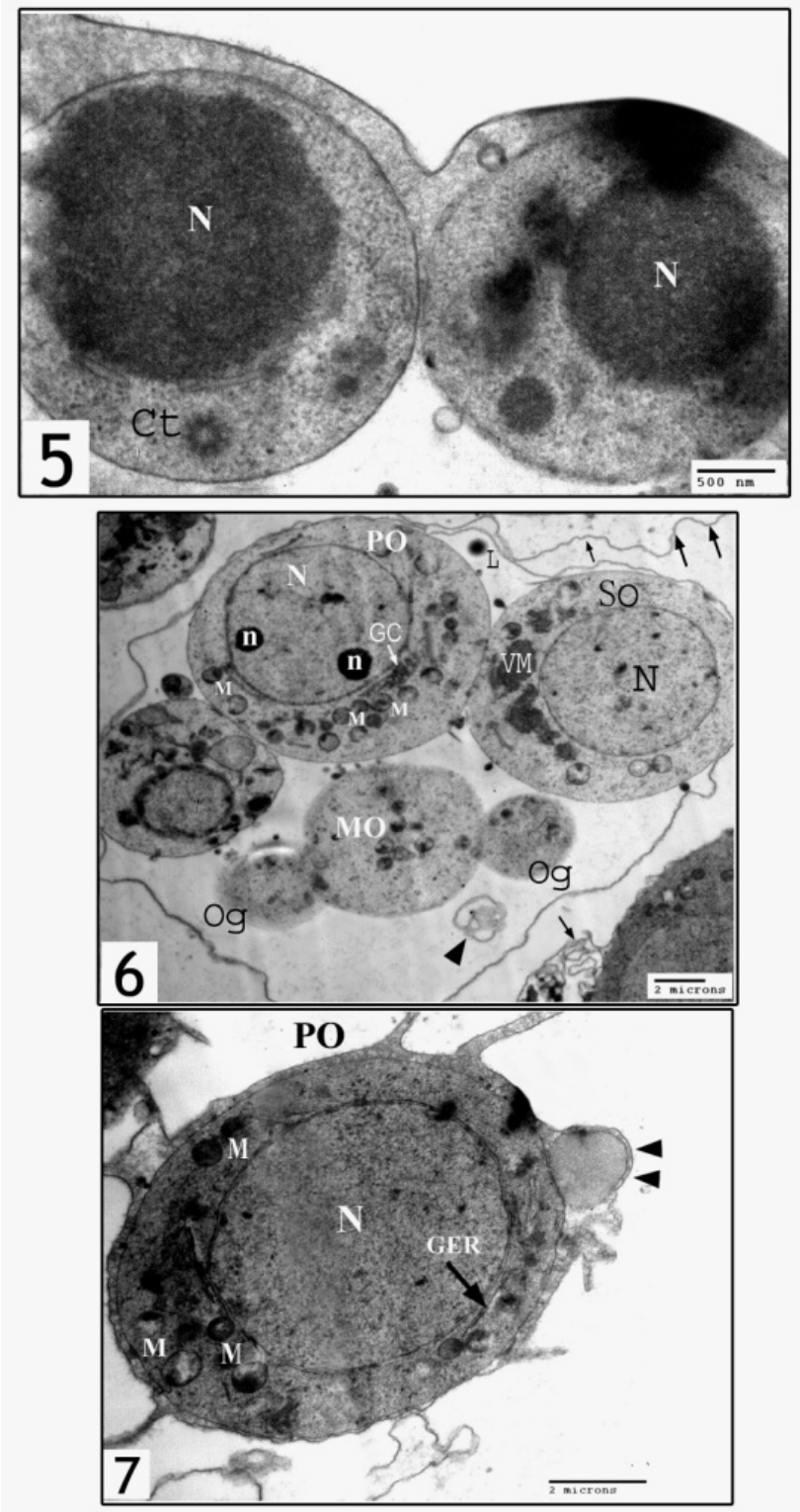


**Figure 2. (5)** Newly formed two oogonial cells surrounded by syncytial cytoplasm, that contains lipid droplet. One centeriole and mitochondria located within the cytoplasm. Scale bar =500nm. **(6)** Acluster of oogonia; primary; secondary and mature oocytes within interstitial cytoplasm, that contains lipid droplet; myelin - like bodies (arrow heads) and lamellar mesh surrounded the cluster. Note that primary oocyte contains numerous mitochondria and elements of Golgi cisternae, Scale bar =2 microns **(7)** Primary oocyte, showing enlarged nucleus, numerous mitochondria, and elements of granular endoplasmic reticulum within the cytoplasm. Interstitial nucleus (arrow heads). Scale bar = 2 micron. 500 nm. BL, basal layer; CG, cortical granules; Ct, centeriole; EP, epithelial projection; FO, fertilized oocyte; GC, Golgi complex; GER, granular endoplamic reticulum; GV, Golgi vesicles; L, lipid; M, mitochondria; MO, mature oocyte; M**v**, microvilli; N, nucleus; n, nucleoules; Og, oogonia; PO, primary oocyte; R, ribosomes; SO, secondary oocyte; SG, shell globules; Sp, spermatozoa; VM, vitelline material.

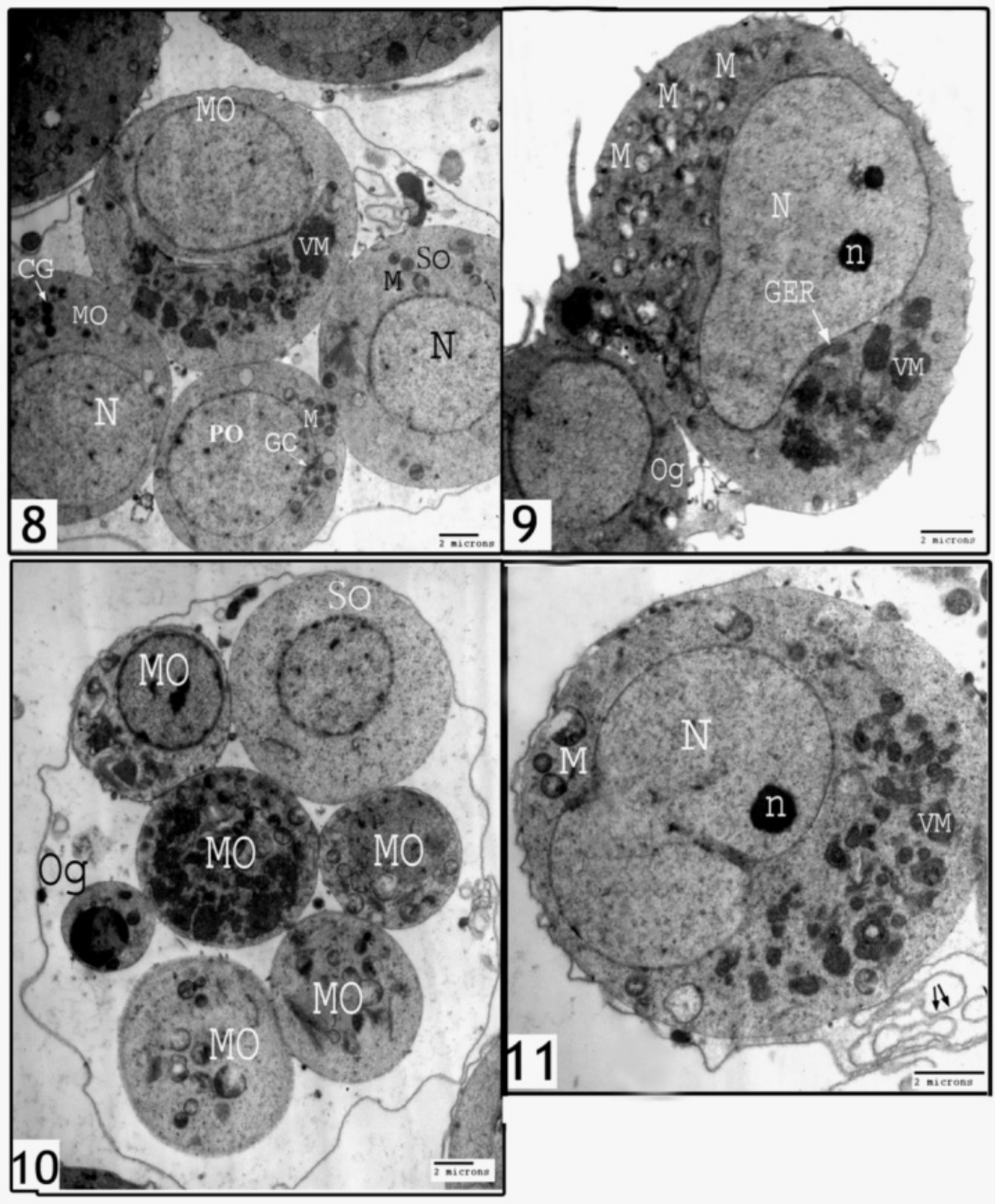


**Figure 3. (8)** A cluster of primary, secondary and mature oocytes within interstitial cytoplasm .Note primary oocyte showing elements of Golgi cisternae, mitochondria and granular endoplasmic reticulum within the cytoplasm, Scale bar = 2 microns. **(9)** Secondary oocyte , with a high cytoplasmic volume containing numerous mitochondria, granular endoplasmic reticulum and fragments of vitelline material. Note division of the nucleolus and elongation of the nucleus. Oogonia in contact with secondary oocyte, Scale bar = 2 microns. **(10)** Aggregation of oogonia ,secondary and mature oocytes surrounded with lamellar membrane, Scale bar = 2 microns. **(11)** Secondary oocyte surrounded by lamellar mesh(arrows). Numerous mitochondria, fragments of vitelline material and begining of division of the nucleus. scale bar = 2 microns. BL, basal layer; CG, cortical granules; Ct, centeriole; EP, epithelial projection; FO, fertilized oocyte; GC, Golgi complex; GER, granular endoplamic reticulum; GV, Golgi vesicles; L, lipid; M, mitochondria; MO, mature oocyte; M**v**, microvilli; N, nucleus; n, nucleoules; Og, oogonia; PO, primary oocyte; R, ribosomes; SO, secondary oocyte; SG, shell globules; Sp, spermatozoa; VM, vitelline material.

[Figure 4 (15)]. Late secondary oocytes are characterized by a greater number of mitochondria, cisternae of GER, Golgi complexes, and a lot of accumulation of electron-dense granules.

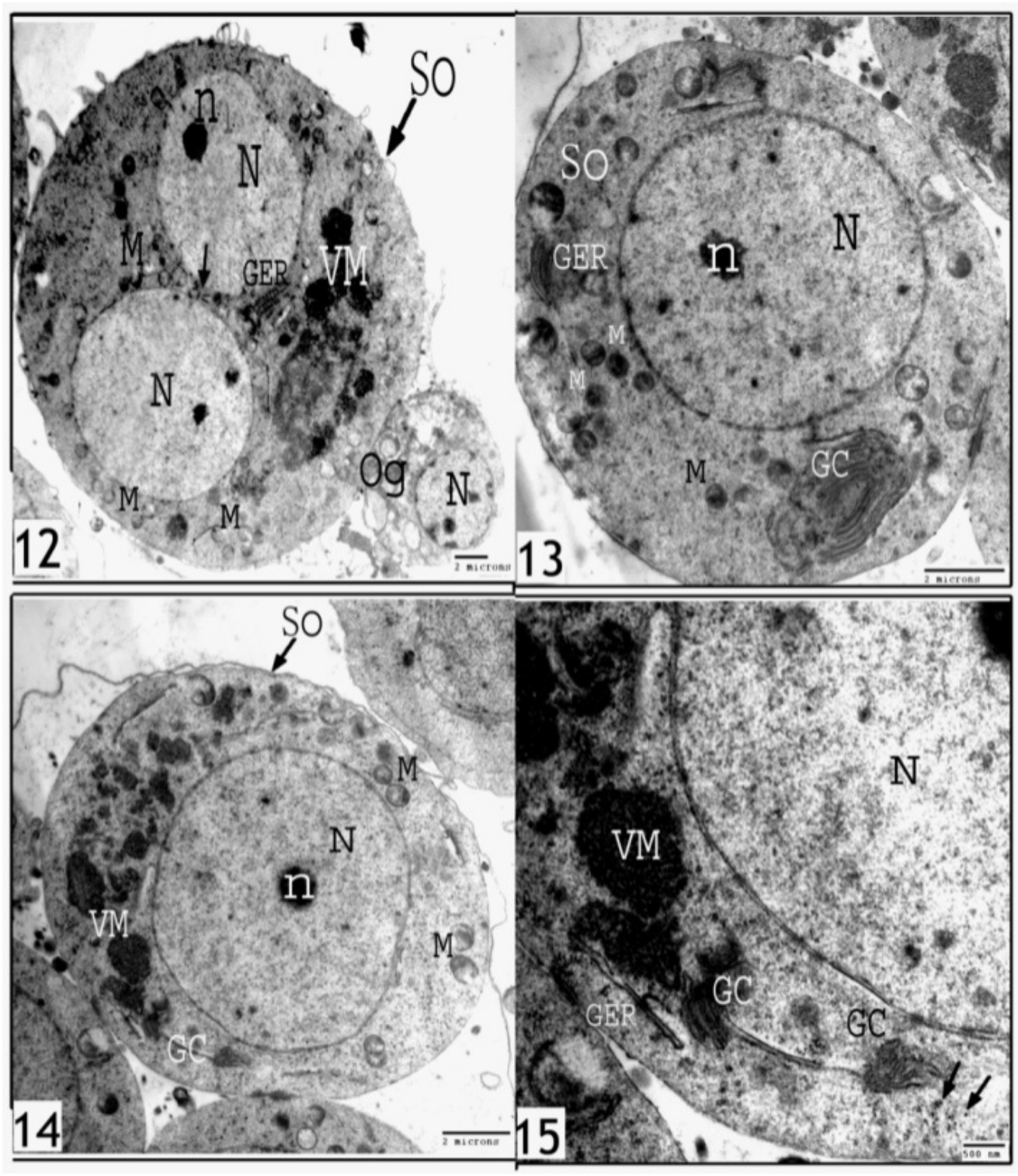


**Figure 4. (12)** Secondary oocyte , showing divided nucleus and one centeriole located between the newly nuclei(arrow).Note oogonia adjacent to the secondary oocyte Scale bar = 2 microns. **(13)** Secondary oocyte , showing well developed Golgi cisternae surrounded with numerous mitochondria and elements of granular endoplasmic reticulum Scale bar = 2 microns. **(14,15)** Larger magnification of secondary oocyte, showing elongate and small cisternae of Golgi complex, enclosing electron dense secretions (arrows) contact with elements of granular endoplasmic reticulum and vitelline materials. Scale bar= 2microns, 500 nm. BL, basal layer; CG, cortical granules; Ct, centeriole; EP, epithelial projection; FO, fertilized oocyte; GC, Golgi complex; GER, granular endoplamic reticulum; GV, Golgi vesicles; L, lipid; M, mitochondria; MO, mature oocyte; M**v**, microvilli; N, nucleus; n, nucleoules; Og, oogonia; PO, primary oocyte; R, ribosomes; SO, secondary oocyte; SG, shell globules; Sp, spermatozoa; VM, vitelline material.

**Mature oocytes**

Mature oocytes are about 22-28 × 14-19 µ in size. They are located more centrally within the ovarian follicles. Their nucleus is eccentric and contains a well-defined nucleolus. At this stage, the cytoplasm is full of vitelline materials, mitochondria and a few lipid droplets [Figure 5 (number 16)]. Synaptonemal complexes within the early stages of the mature oocyte nucleus are observed [Figure 5 (number 17)]. The vitelline material represents mostly the types of cell inclusion; it almost occupies about half of the mature oocyte [Figures 5 (number 17-19)]. A large number of mitochondria and a well developed cisternae of golgi complexes, with its secretions are observed beside the nucleus [Figures 5 (numbers 18,19)] .Spherical cortical granules appear in clusters in mature oocyte [Figure 5 (numbers 20, 21)].

The surface of the plasma membrane detaches to form thin branched lamellae parallel to the surface of the oocyt called lamellar mesh. The main components of the late stage of mature oocyte are the vitelline materials, lipid droplets and cortical granules [Figure 6 (number 22)].

**Follicular oviduct**

A single duct arises from each ovarian lobules and its lumen is filled with mature oocytes. The electron-lucent epithelial lining of these ducts is deeply penetrated by numerous long, branched invaginations of the basal plasma membrane along which are small islets of electron-dense cytoplasm filled with free ribosomes. The ducts are enclosed by a basal matrix continuous with that of the ovarian follicles and peripheral muscles. Mature oocytes within the lumen of the follicular oviducts are usually arranged in a double row. At this stage, most of the cortical granules are adjacent to the oocyte plasma membrane in clusters. Mitochondria, golgi vesicles and a lot of golgi cisternae are located along the surface of the mature oocyte; the vitelline inclusions fill most of the ooplasm [Figure 6 (number 23)].

**Oocapt and main oviduct (proximal part of fertilization canal)**

A muscular sphincter (oocapt) surrounds the junction between the ovarian receptacle and the distal oviduct. It is characterized by a well-developed, closely packed musculature beneath the oocapt epithelium [Figure 6 (numbers 24, 25)]. The latter is a prolongation of the epithelium of the ovarian receptacle and has the same structure. The luminal wall of the oocapt and distal oviduct (fertilization canal) bears cilia and lamellae along its length [Figure 6 (numbers 24, 25)]. Free cortical granules are scattered within the lumen of this duct. The matrix is filled with numerous sperms which penetrate the mature oocyte with their apical cones. There are numbers of mature and fertilized oocytes in the same duct. The mature oocytes in this region are characterized by the presence of many lipid droplets, numerous mitochondria and cortical granules [Figure 6 (numbers 24, 25)].

**Distal part of the fertilization canal**

The luminal wall of the distal part of fertilization canal bears elongated cilia and it contains different stages of fertilized oocytes, that show different stages of vitellocyts with heterogeneous shell-globule clusters; large lipid droplets; compact elongate, peripherally located nuclei with prominent nucleoli and several dense heterochromatin islands in their karyoplasm. All cell organelles (Golgi cisternae, mitochondria and endoplasmic reticulum) are embedded in the remaining cytoplasm [Figure 7 (numbers 26-29)].

**Interstitial syncytium**

The intercellular spaces between the loosely packed oocytes are filled with the processes of interstitial cytoplasm. The interstitial nuclei are usually located in the peripheral area of the follicle [Figure 2 (number 7)]. The long, flattened cytoplasmic processes contain mitochondria,lipid droplets, myelin-like bodies and large, irregularly shaped accumulations of moderately electron-dense homogeneous material [Figures 2, 3, 4 and 5 (6, 8, 10, 11, 16, 20)].

**DISCUSSION**

The general pattern of oogenesis in adult *C. polycantha* is similar to that reported for other species of lower cestodes, that is, gyrocotylideans (Xylander, 1987), amphilinideans (Xylander, 1988), spathebothriideans (Bruňanska et al., 2005; Poddubnaya et al., 2005a, 2006), tetraphyllideans (Mokhtar-Maamouri and Świderski, 1976), caryophyllideans (Mackiewicz, 1968; Świderski and Mackiewicz, 1976; Poddubnaya et al., 2003; Świderski et al., 2004a, b, 2009) and bothriocephalidean (Świderski et al., 2013). The maturation of oocytes of *Cotugnia* follows the pattern described in the other Davaineidae. They increase in size and change their cytological characteristics from poorly differentiated cells (oogonia and primary oocytes) to secondary and mature oocytes. Early stages of mature oocytes are characterized by synaptonemal complexes in the nucleus, and their subsequent disappearance is typical in mature oocytes. This indicates that the oocytes enter the diplotene phase while still in the ovary (Holy and Wittrock, 1986). The development of GER and golgi complexes within the cytoplasm of growing oocytes is as

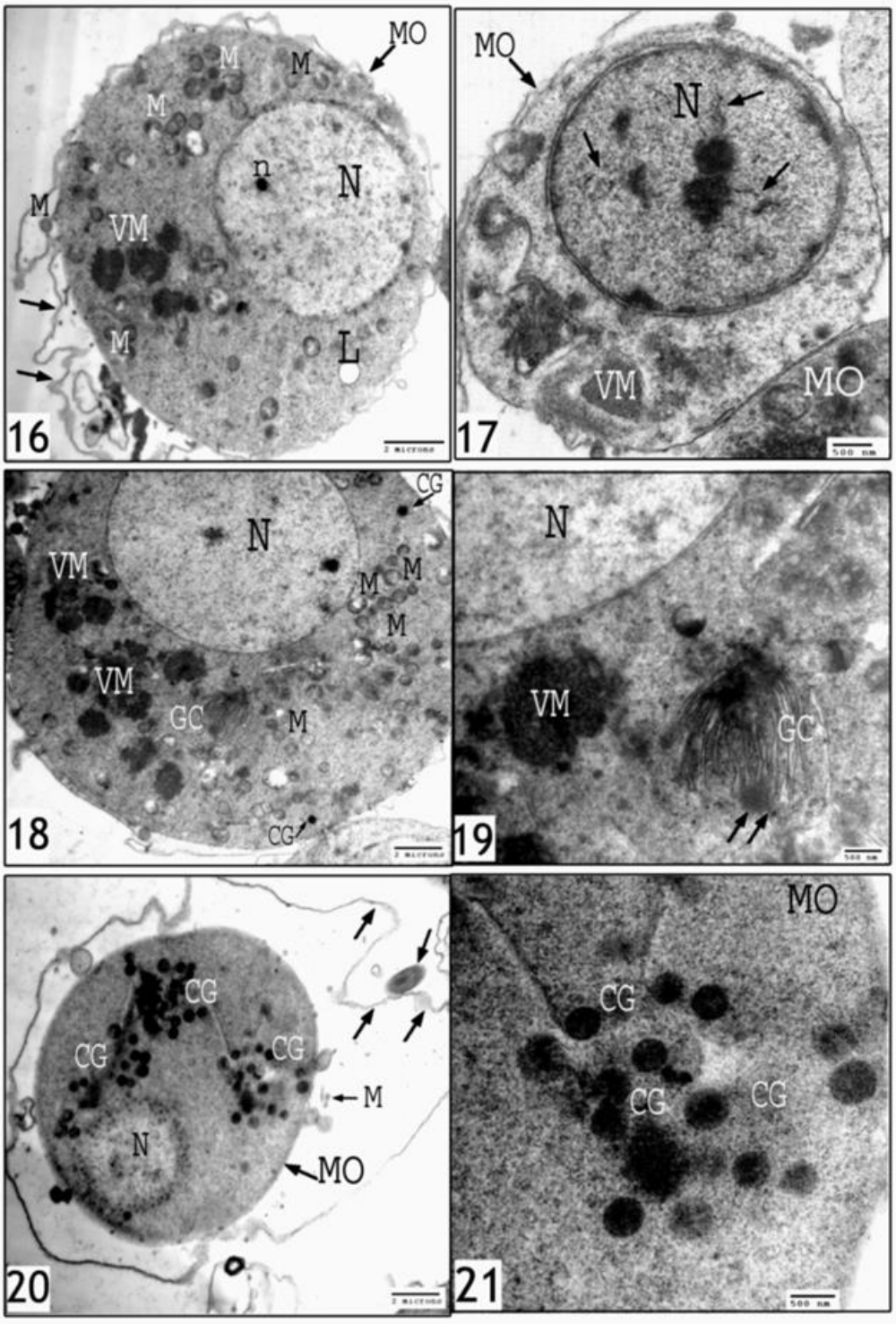


**Figure 5. (16)** Early stage of mature oocyte , showing eccentric spherical nucleus ,abundant mitochondria, fragments of vitelline materials and a few of lipid droplets.Note lamellae that surrounds the outer surface of mature cell(arrows) and interstitial mitochondria. Scale bar = 2microns. **(17)** Early stage of mature oocyte, showing accumulation of vitelline material and synaptonemal complexes are visible within the nucleus Scale bar = 500nm. **(18)** Mature oocyte with numerous mitochondria, Golgi cisternae, vitelline material located adjacent to Golgi cisternae; and cortical granules Scale bar = 2 microns. **(19)** Mature oocyte,showing an accumulation of Golgi cisternae enclosed secretory granules (arrows) Scale bar = 500nm. **(20, 21)** An aggregation of cortical granules within ooplasm of mature oocyte, that surrounded by interstitial mitochondria and myelin – like bodies (arrows). Scale bar = 2 microns, 500 nm. BL, basal layer; CG, cortical granules; Ct, centeriole; EP, epithelial projection; FO, fertilized oocyte; GC, Golgi complex; GER, granular endoplamic reticulum; GV, Golgi vesicles; L, lipid; M, mitochondria; MO, mature oocyte; M**v**, microvilli; N, nucleus; n, nucleoules; Og, oogonia; PO, primary oocyte; R, ribosomes; SO, secondary oocyte; SG, shell globules; Sp, spermatozoa; VM, vitelline material.

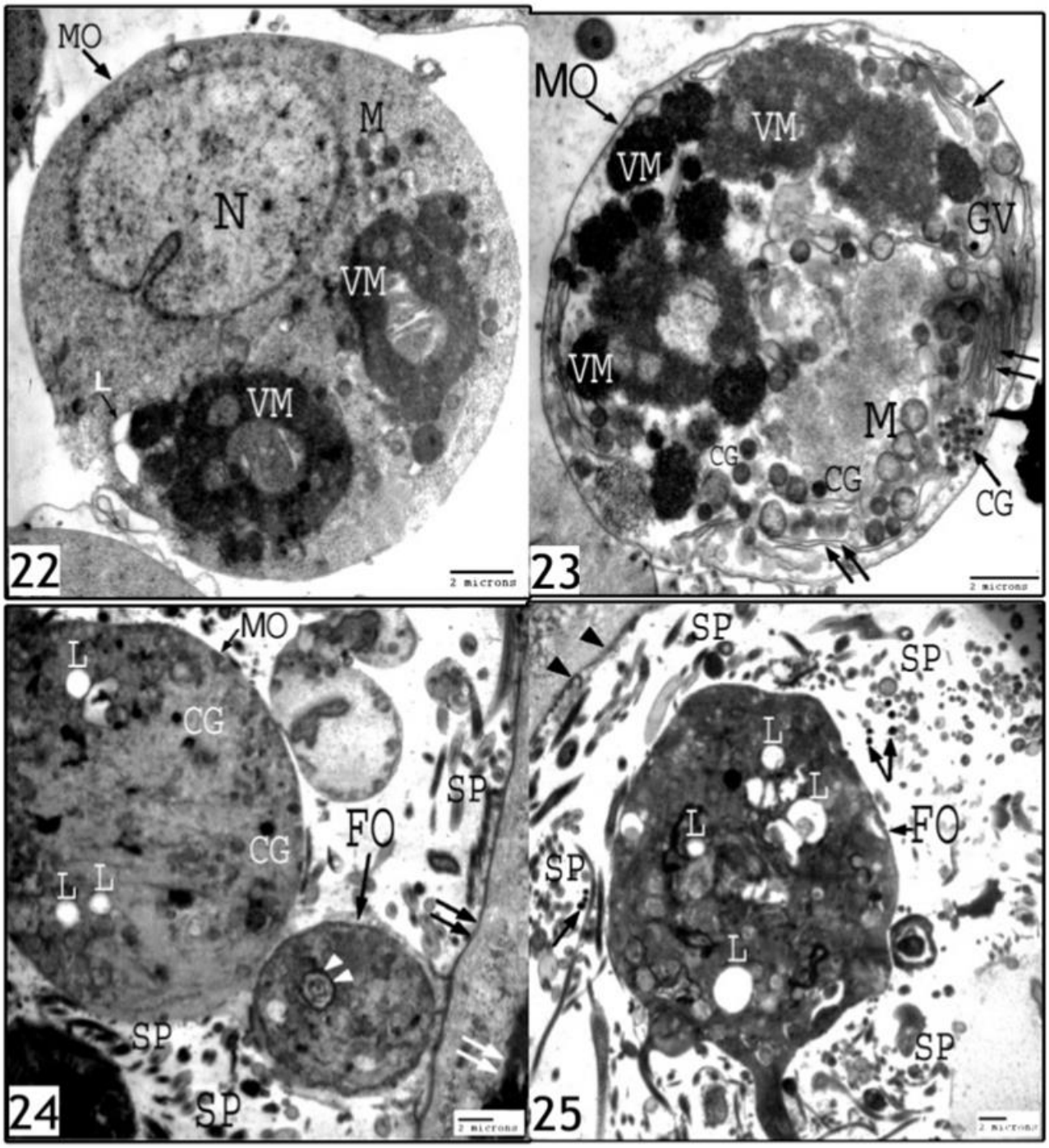


**Figure 6. (22)** Late stage of mature oocyte , showing a condensed vitelline materials,numerous mitochondria and lipid droplets Scale bar = 2microns. **(23)** Mature oocyte within folliculer oviduct lumen, showing clusters of cortical granules ;Golgi cisternae(arrows); Golgi complex vesicles with electron dense granules inside .Note numerous mitochondria and vitelline materials Scale bar = 2microns. **(24)** Epithelial wall of the proximal part of fertilization canal with muscles, short lamellae and cilia( arrows) ,contains mature oocyte surrounded by numerous sperms, that penetrate the surface of mature cell . Fertilized oocyte rest on the wall of fertilization canal and penetrated with sperm (arrow heads). The mature oocyte contains numerous mitochondria ,cortical granules and lipid droplets Scale bar = 2microns. **(25)** Lumen of fertilization canal contains free cortical granules( arrows) and fertilized oocyte , that contains numerous lipid droplets and surrounded by numerous sperms . Short lamellae and cilia( arrow heads) of epithelial wall of fertilization canal.Scale bar = 2 microns. BL, basal layer; CG, cortical granules; Ct, centeriole; EP, epithelial projection; FO, fertilized oocyte; GC, Golgi complex; GER, granular endoplamic reticulum; GV, Golgi vesicles; L, lipid; M, mitochondria; MO, mature oocyte; M**v**, microvilli; N, nucleus; n, nucleoules; Og, oogonia; PO, primary oocyte; R, ribosomes; SO, secondary oocyte; SG, shell globules; Sp, spermatozoa; VM, vitelline material.

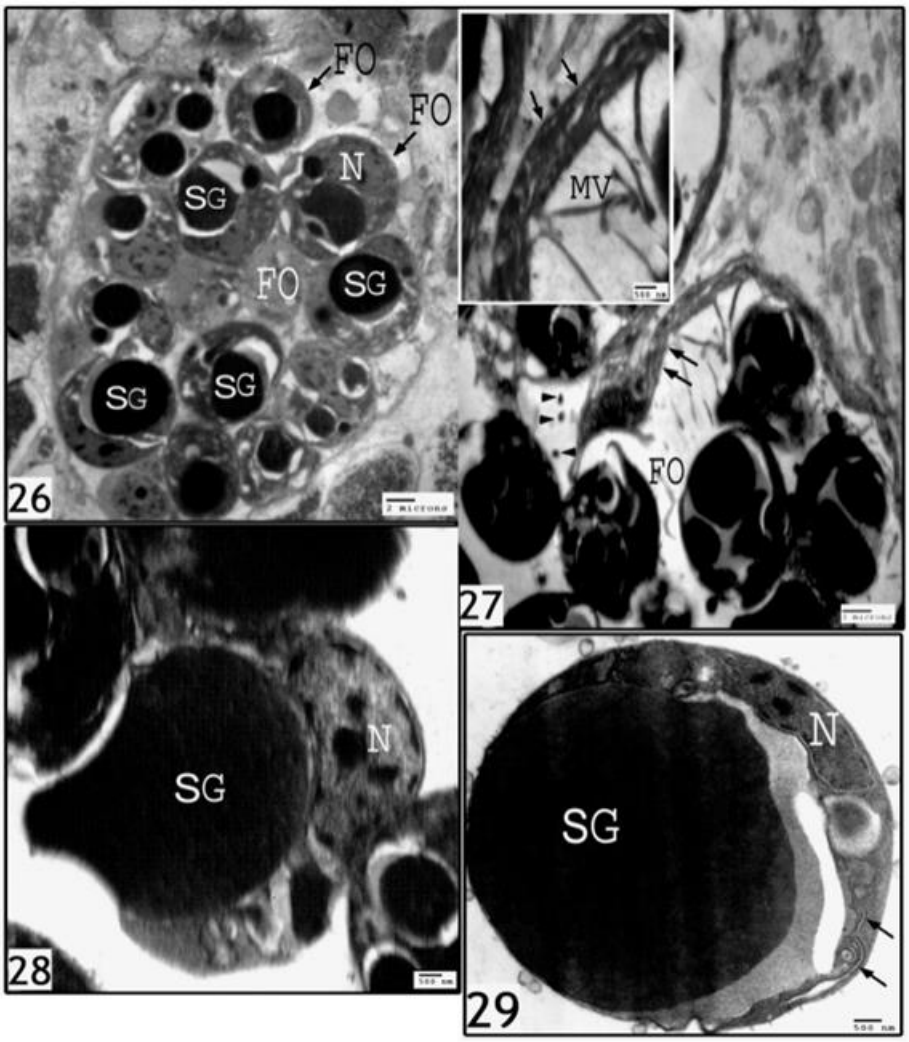


**Figure 7. (26)** Aggregation of fertilized oocytes within lumen of the distal part of fertilization canal , showing enlarged shell globules of vitellocytes fill most of the cell, lipid droplets and peripherally located nucleus scale bar = 2 microns. **(27)** Epithelial wall of the distal part of fertilization canal (arrows) with apical lamella and cilia,the lumen is crowded with numerous fertilized oocytes and free cortical granules(arrow heads) .Insert show heigh magnification of epithelial wall with muscles and elongated cilia scale bar = 2 microns. **(28, 29)** Enlarged fertilized oocytes, showing shell globules fill most volume of the cell; peripherally compressed nucleus; Golgi cisternae(arrows);and granuler endoplasmic reticulum. Scale bar = 500 nm. BL, basal layer; CG, cortical granules; Ct, centeriole; EP, epithelial projection; FO, fertilized oocyte; GC, Golgi complex; GER, granular endoplamic reticulum; GV, Golgi vesicles; L, lipid; M, mitochondria; MO, mature oocyte; M**v**, microvilli; N, nucleus; n, nucleoules; Og, oogonia; PO, primary oocyte; R, ribosomes; SO, secondary oocyte; SG, shell globules; Sp, spermatozoa; VM, vitelline material.

a result of the production of electron-dense granules and lipid droplets and subsequent migration of these granules to the cortical ooplasm, where they form a cluster. Oocytes of many Davaineidae contain cytoplasmic inclusions, such as lipids and cortical granules.

In the present study, *C. polycantha* shows a significant variation in the products of mature oocyte and variations in the ultrastrucure of developing cells during the oogenesis than cestodes like *Killigrewia delafondi* (Taeleb and Abdel-Moaty, 2011*)*. Vitelline materials appear early in secondary oocytes, and there are no previous references of this phenomenon.

The late appearance of lipid droplets in mature acute stages is one of the most striking features, as it represents an additional energy reserve for subsequent development of the embryo. Biosynthesis of lipids depend

on the host's fatty acids (Buteau et al., 1971; Nakagawa et al., 1987). Because of this relationship, the fatty acid composition of custards is quite similar to that in their immediate environment within the host (Beach et al., 1975), and the host's fatty acids are derived from its food chain. Consequently, it can be concluded that the quantity of lipids in a parasite's tissue is generally host-related. In *Killigrewia delafondi* (Taeleb and Abdel-Moaty, 2011), lipids represent the most abundant cell inclusion and appear at an earlier stage of oogenesis and vitellogenesis (Świderski and Mokhtar, 1974; korneva, 2001; Świderski et al., 2011). Furthermore, the histochemical study of Moczoń (2006) on the accumulation and utilization of lipids during the development of the cysticercoid metacestode of the cyclophyllidean *Hymenolepis diminuta* confirmed their important role in cestode morphogenesis.

On the other hand, Poddubnaya et al. (2005b) reported that the mature oocytes within the lumen of the follicular oviducts are full of cortical granules which are adjacent to the oocyte plasma membrane as clusters. The structure of the cortical granules has been shown to be useful in phylogenetic studies of the Platyhelminthes (Sopott-Ehlers, 1991).

Spherical homogeneously accumulated cortical granules that appeared in the sites within the ovary closely resemble those described by Poddubnaya et al. (2005a, b) in the spathebothriidean *Diplocotyle olrikii* and *Cyathocephales truncates* but exceed that described in *Killigrewia delafondi* (Taeleb and Abdel- Moaty, 2011).

Awad and Probert (1990) described another type of cortical granules formed of a dense core with a number of outer lamellae in some trematodes. The variations in the morphology of different worms may indicate variations in chemical composition as reported by Poddubnaya et al. (2005a).

Cortical granules have been reported to be composed of protein and carbohydrates and it is suggested that they represent nutritive structures (Gresson, 1964; Anderson, 1968; Boyer, 1972). Spot-Ehlers (1991) reported that the structure of cortical granules is useful in phylogenetic studies of Platyhelminthes. Grant et al. (1977), Hathaway (1979), Justine and Mattei (1984), Cifrian et al. (1993), Poddubnarya et al. (2007) and Poddubnaya et al. (2010) revealed that small cortical granules (less than 3.0 (m) are present in Digenea, Aspidogastrea, Gyrocotylidea and Cestoda. It also plays a crucial role in the so-called "cortical reaction" and is considered to prohibit polyspermy in some animals (Tyler, 1965; Guraya, 1969; Gray et al., 1976; Świderski, 1976; Świderski and Conn, 1999; Świderski et al., 2004). It is likely that the muscular sphincter controls the passage of mature oocytes, from the ovary receptacle into the fertilization canal.

Syncytial interstitial tissue is present in the ovarian follicles; it fills the cytoplasm spaces between the oocytes.

Such interstitial cells are supposed to be responsible for the transport of nutrients and energy sources (Gresson, 1964; Orido, 1987; Conn, 1993; Świderski and Xylander, 2000; Podvyaznaya, 2003; Poddubnaya et al., 2005a, 2007; Conn et al., 2009).

A number of myelin-like bodies in the ovarian interstitial cytoplasm are similar to that reported in *K. Delafondi*, and *Didymobothrium Rudolph II* (Taeleb and Abdel- Moaty, 2011; Poddubnaya et al., 2007). Supporting interstitial tissue has also been described in the ovary of the trematode *Paragonimus ohirai* (Orido, 1987), *Diplocotyle olrikii* (Poddubnaya et al., 2005a) and the cestode *Gyrocotyleurna* (Puddobnaya et al., 2010).

Davaineidae resembles the neodermates as the sites are usually transported from the ovary to the oviduct through the oocapt. The oocapt ultrastructure has been investigated in digeneans, monogeneans and cestodes (Gupta et al., 1983; El-Naggar et al., 1990; Podvyaznaya, 2003; Davydov et al., 1994; Galaktionov and Dobrovolskij, 2003; Poddubnaya et al., 2007; Poddubnaya et al. 2010).

A previous study on *Gyrocotyle* has shown that the oviduct is represented by multiple ducts that form an ovarian receptacle for mature oocytes prior to forming the fertilization canal (Poddubnaya et al., 2010). This is similar to our finding as muscular sphincter (oocapt) surrounds the junction between the ovarian receptacle and the distal oviduct. It is characterized by a well-developed, closely packed musculature beneath the oocapt epithelium, which shows a prolongation of the epithelium of the ovarian receptacle. The luminal wall of the oocapt and distal oviduct (fertilization canal) bears cilia and lamellae along it, with various lengths (El-Naggar et al., 1990; Poddubnaya et al., 2005b; Poddubnaya et al., 2010).

The luminal wall of the distal part of fertilization canal bears elongated cilia and contains different stages of fertilized oocytes that show different stages of vitellocyts with heterogeneous shell-globule clusters (Poddubnaya et al., 2010).

The fertilization process of *C. polycantha* as the matrix filled with numerous sperms which penetrate the mature oocytes with their apical cones is been recored for the first time, as well as the fertilized oocytes that show sperm axoneme in the middle.

## Conflict of Interests

The author(s) have not declared any conflict of interests.

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