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Horticulture Performance and Genetic Diversity Based on RAPD Marker for some Egyptian Mandarin Cultivars

Hala M. El-Khayat[1] *and Dalia G. Aseel*[2*]

[1]Horticulture Research Institute, A.R.E., Egypt

[2]Plant Protection and Biomolecular Diagnosis Department, Arid Lands Cultivation Research Institute, City of Scientific Research and Technological Applications (SRTA,City), New Borg El-Arab City, 21934, Egypt.

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Abstract: Four mandarin (Citrus reticulata, Blanco) cultivars; Fremont, Murcott, Balady, and Ponkan grown in calcareous soil were evaluated for their growth, production, fruit quality and genetic characteristics during the growing seasons of 2014 and 2015. The obtained data showed that, Fremont mandarin has the highest leaf total carbohydrate and chlorophyll in the second season and leaf magnesium, while Murcott mandarin has the highest leaf nitrogen and phosphorus in the first season and leaf total carbohydrate in both seasons. In the meantime, Balady mandarin has the highest leaf phosphorus, fruit number and acidity in both seasons and fruit total soluble solid in the second season. Ponkan madarin has the highest vegetative growth (tree height, trunk circumference, canopy circumference) leaf potassium, leaf calcium, yield, fruit length, fruit diameter, fruit total soluble solid in the first season and Vc in both seasons. We analyzed the genetic diversity of four mandarin cultivars to understand their diversity and relationships within the four cultivars. Genetic analysis was conducted using data from RAPD marker, PCR, sequences of a malate dehydrogenase gene and phylogenetic Neighbor joining were conducted. The results of genetic diversity for four mandarin cultivars were used phylogenetic tree for both RAPD and specific PCR of MDH gene which showed close similarities of the three mandarin cultivars; Murcott, Balady and Ponkan in same cluster, while, another cluster was consisted only Fermont cultivar. Generally, using RAPD markers with whole genomic DNA provided the investigation the chance to match, distinguish Citrus cultivars with specific fragments or alleles and was able to assess genetic diversity among citrus cultivars. It can be suggested that the usage of RAPD markers is capable in identification fractional genome in breeding programs. Random primers can differentiate, estimate, characterize and determine genetic diversity and phylogenetic relationship (genetic similarity) among four mandarin Citrus reticulate, Blanco) cultivars, Fremont, Mucott, Balady and Ponkan.

Keywords: Mandarin cultivars, RAPD-PCR, MDH gene.

1 Introduction

Citrus production comprises the largest fruit sector in Egypt. Egypt has a great potential for citrus production when its ecological and other characteristics were considered. According to, Static's of Agricultural Production Requirements [1]. Production of Egypt citrus is 3.181.000 t coming from 2,109,000 t of orange, 732,000 t mandarins, 297, 000 t lemon and 43,000 t grapefruit. In the past, research has been mainly focused on relatively Balady mandarin orchards in clay soil in Nile Delta. During the recent years, the programs of agricultural development in Egypt aim to increase the cultivated areas of mandarin cultivars especially in the newly reclaimed land Therefore, other mandarin cultivars such as Fermont, Murcott and Chinese (Ponkan) were introduced to be planted under the Egyptian conditions and in different soil types. Fermont is a crossing between Clementine and Ponkan mandarins it is an early maturing variety. Its tree has a moderate vigor with attractive brilliant reddish–orange easy to peel fruits [2]. The Murcott cultivar is most likely a cross between tangerine and sweet orange. The trees are vigorous and bushy in shape. Its fruit has yellow peel, deep orange flesh, 10-20 seed and is exceptionally sweet but difficult to peel. Murcott mandarin is commercially harvested during January to March [3].

Balady was the first mandarin introduced into the Mediterranean basin from China in 1805 via England, to

*Corresponding author e-mail: daliagamil52@gmail.com

Malta and finally to Italy before being distributed throughout the region and later world. The tree is slow growing and of medium size with drooping branches, is nearly thorn less, and has very small narrow leaves. It is fairly cold –resistant and strongly inclined to alternate-bearing. The fruit is small to medium with yellowish – orange thin easy to peel rind. Additionally, the cultivar (Ponkan) which is known as Chinese in Egypt. This is the most widely grown mandarin in the world, being common throughout south China and southern Japan, the Philippines (Batangas) and India (Nangpur suntara) it is also popular mandarin in Brazil. The Ponkan tree is vigorous and of medium size for mandarin. Ponkan fruit is large for a mandarin, averaging around 80mmin diameter and has a very oblate shap.It is one of the easiest mandarin to peel and the rind oil has a district aroma similar to the Mediterranean mandarin [2].

The molecular markers have been followed as a valuable and precise strategy to physiological and molecular studies on Citrus. Random amplified polymorphic DNA (RAPD) has been employed most widely. So, RAPDs have been used for cultivar identification, genetic diversity, genetic mapping assessment and other breeding programs [4]. Moreover, the genus Citrus, information dealing with the generation of genomic tools and resources on the progress in molecular biology and genetics of development [5]. Furthermore, Genetic diversity assessment in plants has now become far more simple, cost effective, reliable and reproducible; thanks to the advent of PCR-based DNA marker techniques such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeat (ISSR), simple sequence repeats (SSR), directed amplification of minisatellite DNA (DAMD) [6]. RAPD is a simple and fast DNA molecular marker technique to randomly amplify DNA fragments under low-stringency conditions by short oligonucleotides [7]. RAPD has been widely used for taxonomy studies, differentiating hybrids in *Citrus* breeding program, identify mutation, phylogenetic analysis, genetic diversity, mapping and molecular assistant selection [8, 9, 10].

Malate dehydrogenase belongs to the A group of dehydrogenases, which constitute a gene family of NAD (P)+ dependent conserved enzymes that are ubiquitously found in plants, animals, fungi and bacteria [11]. Malate is a central metabolite that is essential for cellular metabolism and an important intermediate of the tricarboxylic acid cycle [12]. However, several MDH genes have been identified in a number of plants, including Arabidopsis [13], maize [14], apples [15] and cotton [16]. Functional studies revealed that MDHs were involved in the growth and development of plant cells and played a crucial role in various plant stress responses, such as leaf respiration [13], embryo development [17] and tolerance to cold and salt stress [18]. The recent identification of different malate protein channels in several plant tissues and the analysis of transgenics with varied malate metabolisms have shed new light on its broader importance for cellular functions [19]. Accordingly, the present evaluation was under taken in order to differentiate the mandarin cultivars; Balady, Fremont, Murcott and Ponkan grown in calcareous soil in respect of tree growth, leaf chlorophyll and mineral content, as well as tree yield, fruit quality and genetic characteristics.

2 Experimental Section

2.1 Materials

2.1.1 Plant Materials

The present study was undertaken in 2014 and 2015 growing seasons in private orchard in Bostan region, El-Behera governorate, Egypt in order to evaluate four mandarin (*Citrus reticulate*, Blanco) cultivars; Fermont, Murcott, Balady and Chinese (Ponkan) planted in 2007 in calcareous soil as shown in Figure (1). Twenty trees (4 cultivars x 5 rep1) in a randomized complete block design (RCBD) with one tree per replicate were selected as uniform as possible in growth, productivity and appearance for the present evaluation study.

2.2 Methods

2.2.1 Vegetative Growth

Vegetative growth as the tree height, trunk circumference and canopy circumference were measured in both seasons. Tree yield was estimated as number and weight (Kg) of fruits per tree at the commercial harvest date of each cultivar.

2.2.2 Leaf and Fruit Analysis

In addition, a sample of 8 leaves was randomly selected from the middle part of non-fruiting shoots of each tree in September of both seasons in order to determine leaf total Chlorophyll using Minolta SPAV chlorophyll meter model [20]. Then the rest of the same leaf sample was washed with tap and distilled water, oven dried at 65-70°C to a constant weight. The dried leaf tissues were grounded and digested with sulphuric acid and hydrogen peroxide as mentioned by [21].

2.2.3 Mineral Composition

Suitable aliquots were taken for the determination of total nitrogen and phosphorus calorimetrically according to [22, 23], respectively. Potassium was determined by flame photometer as described by [24]. Calcium and magnesium was determined by atomic absorption according to [25].

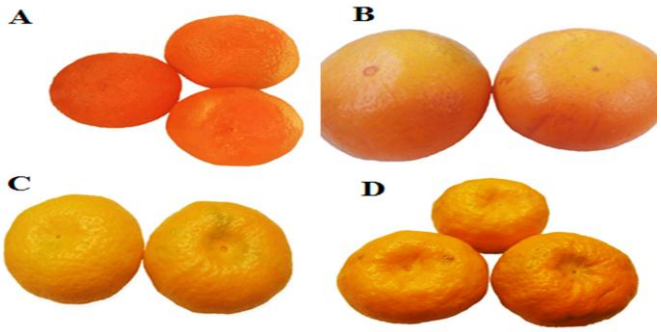

Fig. 1: Mandarin (*Citrus reticulate*, Blanco) cultivars in Egypt, A; Fermont, B; Murcott, C; Balady and D; Ponkan.

In addition, for measuring fruit quality characters, a sample of 10 fruits was randomly collected from each tree at harvest date fruit length and diameter were calculated and the percentage of fruit total soluble solids (TSS) was measured using a hand refractometer. Fruit acidity (%) as citric acid and vitamin C (mg ascorbic acid /100 ml juice) content was determined according to A.O.A.C. [26].

2.2.4 Statistical Analysis

Finally, all data obtained were one way statistically analyzed [27] with the SAS version [28].

2.2.5 DNA Extraction from Plant Leaves

The leaves were grounded to a fine powder in liquid nitrogen and the DNA of *Citrus reticulata* plant were isolated from four cultivars; Fermont, Murcott, Balady and Chinese (Ponkan) leaves using I-Genomic Plant DNA Extraction Mini Kit (INRTON) according to manufacturer's instructions.

2.2.6 RAPD-PCR and Electrophoresis

Nine random primers were used to differentiate fingerprint of the isolated from four cultivars of *Citrus reticulata* which were under our study by using DNA. Sequences of primers were illustrated in (Table 5). The PCR reaction mixture was carried out in a final volume of 25µL, consisting of (8.8 µL of Sterile Milli Q water; 5µL of 5 x PCR reaction green buffer; 2.5µL of 50 mM-MgCl2; 2.5µL of 25mM-dNTPs; 5µL (50 pmol/µL) of each arbitrary primers; 0.2 (5U/µL) Taq polymerase and 1µL of DNA). The applied PCR program was performed as follows: initial denaturation at 95°C for 5 min.; 40 cycles at 95°C for 1 min.; annealing ranged at 28-30°C for 1 min and extension at 72°C for 1min. A final extension step at 72°C for 10 min. PCR amplifications were separated on agarose gel electrophoresis [29].

2.2.6.1 RAPD Analysis and Phylogenetic Relationships

Bands of DNA fragment were scored manually for all the samples studied as present (1) and absent (0). Level of marker polymorphism according to the various molecular techniques and data were analyzed with program STATISTICA(R) software version 5. And dendogram displaying relationships of four citrus cultivars was constructed using the unweighted pair group's method arithmetic average (UPGMA).

2.2.7 Detection and Identification of Malate Dehydrogenase (MDH) Gene, Sequencing and Phylogenetic Analysis

A pair of degenerate oligonucleotide primers were corresponding to (forward; 5`-GCTCCTGTGGAAGAGACCC-`3 and reverse; 5`-TCCAGAGATGACCAAAC-3′) as described previously [30] was used to amplify the DNA fragments of MDH gene of four cultivars; Fermont, Murcott, Balady and Chinese (Ponkan) citrus plants. PCR reaction mixture was carried out in a final volume of 25µl, consisting of 1µL DNA template, 5µL of 5x buffer, 2.5µl $MgCl_2$, 2.5µL dNTPs, 1 µL of each primer (10 pmol/ µL), 1U Taq DNA polymerase (Promega, USA) and up to volume nuclease-free water. PCR was programed with initial denaturation at 95°C for 5 min and 30 cycles at 95°C for 1 min.; annealing at 55°C for 1 min and extension at 72°C for 1min. with final extension step at 72°C for 10 min. Finally, 5µl of each PCR product was migrated on agarose gel electrophoresis [31]. DNA sequence for the MDH gene for three mandarin (*Citrus reticulate*, Blanco) cultivars; Fermont, Murcott and Chinese were performed by Macrogene Company (Korea). The obtained DNA nucleotide sequences were analyzed using NCBI-BLAST (http://bast.ncbi.nlm.nih.gov/Blast.cgi) for confirming the identity of the obtained sequences. The sequences were used for comparison using MEGA 4 [32], and phylogeny was tested with bootstrap method. The phylogenetic tree was analyzed and generated based on neighbor-joining statistic method.

3 Results and Discussion

3.1 Vegetative Growth

Regarding to the data of both season in Table (1), tree height of different cultivars ranged from 251.6 to 340cm. Chinese (Ponkan) mandarin had significantly more tree height in comparison with the other three cultivars. In the meantime, the Murcott trees tended to be significantly higher than the Balady cultivar. As for the trunk cross section of the examined trees, it ranged from 28 to 51cm. The Chinese (Ponkan) mandarin trees had significantly higher trunk circumference than the Balady, Fermont and Murcott trees. In addition, tree canopy circumference ranged from 540 to 827 cm. The highest tree canopy was observed in the Chinese (Ponkan) cultivar in comparison with other three cultivars, while, the Balady mandarin trees had significantly higher canopy volume than Murcott and Forment trees in the second season.

Concerning fruit number per tree is presented in Table (1). Balady mandarin showed significantly higher fruit number (547) as compared with all other mandarin varieties in 2014 and Fermont (424) and Murcott (342) cultivars in 2015 Table (1). However, Shamima et al. (2013*)* reported lower fruit number of the kinnow 316, 262 and 268 fruit/tree, respectively).

Tree yield estimated as kg fruit per tree, the Chinese (Ponkan) mandarin recorded the highest yield (87kg/tree) as compared with Balady (73.67kg fruit/tree), Murcott (57kg fruit/tree) and Fermont (51.67kg fruit/tree). However, Balady cultivar had significantly higher yield than the Fermont and Murcott cultivars Tabel (1). Previous studies reported the yield of different mandarin cultivars. The yield of Nagpur' mandarin tree ranged from15.07 to 34.78kg fruit/tree [33]. While, a range of 24.4 to 72.8kgfruit/tree for Nagpur' mandarin tree was reported by [34]. The Kinnow mandarin yield ranged from 23.39 to 32.77kg fruit /tree [35].

3.2 Leaf Analysis

Regarding to the data in Table (2) leaf total carbohydrate: Murcott (8.24 %) contained significantly leaf total carbohydrate as compared with Formont (6.93), Balady (5.9%) and Chinese (Ponkan) (5.94%) cultivars in the first season and Fermont (8.19%), Also, Murcott (8.3%) cultivars contained significantly higher leaf total carbohydrate as compared with Balady (5.78%) and Chinese (Ponkan) (5.24%) in second season. On Washington Navel Orange found that leaf total carbohydrate was7.59% for control plants [36]. On Balady mandarin reported that leaf total carbohydrate was 9.28% and 9.3% for control plants [37].Concerning to total chlorophyll, the data in Table (2) reported that total chlorophyll significantly increased in Chinese (Ponkan) mandarin (73.6 SPAV Unite) than Balady (71.1 SPAV Unite) and Fermont (70.23 SPAV Unite) cultivars in 2014 and significantly increased in Murcott (74.73 SPAV Unite) than Balady (64.63 SPAV Unite) and Chinese (Ponkan) (66.7 SPAV Unite) cultivars in 2015. In another study on sour orange found that the total chlorophyll in leaf was 53.08 and 47.03 for control plants [38].

3.3 Fruit Physical and Chemical Quality Characteristics

Fruit physical and chemical quality characteristics were also recorded for each cultivar and are presented in tables (2 & 3). The Chinese (Ponkan) cultivar recorded the highest fruit length and diameter (5.25 and 6.69cm, respectively) in comparison with all other with the other studied cultivars. In the meantime, fruit diameter of Balady mandarin was significantly higher than fruit diameter of Fermont and Murcott cultivars (Table 2). The fruit diameter ranging from 65.90 to71.58mm in fruit mandarin of Sillanwali, 67.70 to70.96mm in Sargodha and 64.33 to 69.71mm in Bhalwal mandarin cultivars [39]. A fruit length ranging from 4.35 to 4.90 cm and fruit diameter ranging from 4.68 to 5.48cm in the kinnow mandarin cultivar [35].

In addition, fruits of the Chinese (Ponkan) cultivar in 2014 and 2015 had significantly higher vitamin C content (66.67 & 69mg/100ml juice) than fruits of Fermont (38 & 41.33mg /100ml juice), Murcott (38.67 & 41.67mg/100ml juice) and Balady (42.67 & 46 mg/100ml juice). However, the Balady, Fermont and Murcott cultivars did not differ among each other in their fruit vitamin C content (Table 3). Similarly, [40] recorede a vitamin C value of 36.36 and 43.1 mg/100ml juice in Balady mandarin, (31.87and 42.76 mg/100ml juice) in Clementine and (38.94, 40.77 mg/100ml juice) in Chinese mandarin.

Regarding the fruit total soluble solids content (TSS), the data in table (3) showed that in general, the fruits of Chinese (Ponkan) mandarin contained higher TSS (12.03) than Murcott (10.77%) and Balady (11.33%) cultivars in 2014 season. Also, fruits of Balady mandarin contained higher (12.4%) TSS than the Murcott (10.4%) and Fermont (10.77%) cultivars in 2015 season. The TSS percentage was recorded in fruit ranging from 11.3 to 12.11 in Kennow mandarin fruits collected from Sillanwali, 11.2 to 12.57 in Sargodha and from 10.57 to 12.66 in the fruits collected from Bhalwal [41]. Never the less, [33] recorded total soluble solids content in the fruits of Nagpur mandarin ranging from 9.5 to 10.2%.

Concerning fruit acidity, the data in Table (3) indicated that balady mandarin (0.483%) gave higher acidity when compared with Chinese (Ponkan) mandarin (0.417%) in the first season, however no significant differences were found in second season. The acidity in five lots of Kinnow mandarin ranged from 1.01 to1.18 percent in case of Sillanwali, 1.06 to 1.20 percent in fruits taken from Sargodha and 1.08% to 1.21 percent in the fruits of Bhalwal [41]. Nagpur mandarin found that acidity ranged from 1.01% to 1.18% [33]. As for fruit TSS / acidity, fruit TSS/acidity generally tended to be significantly higher in Chinese (Ponkan) mandarin (28.86) than all other studied cultivars (Table 3) in the first season however, no significant differences were found in second season.

Table 1: Evaluation of height of tree trunk across, canopy volume index, fruit number and yield for some mandarin cultivars

Cultivars	Height of tree cm		Trunk circumference cm		Canopy circumference cm		Fruit number per tree		Yield kg per tree	
	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Fermont	252^{cb}	268^{cb}	30.0^{b}	31.3^{b}	600^{b}	650^{c}	413^{c}	424^{b}	52^{c}	53^{d}
Mucott	265^{b}	276.7^{b}	28.0^{b}	30.7^{b}	540^{c}	603^{d}	337^{d}	342^{c}	57^{c}	57^{c}
Balady	245^{c}	260^{c}	27.7^{b}	31.0^{b}	627^{b}	700^{b}	528^{a}	547^{a}	74^{b}	77^{b}
Ponkan	320^{a}	340^{a}	45.7^{a}	51.0^{a}	777^{a}	857^{a}	516^{b}	540^{a}	87^{a}	90^{a}
LSD.05	13.83	14.61	3.71	2.209	28.08	13.685	8.0124	27.684	7.3256	2.8255

* Each value represents the mean of three replicates.
** Values within a column followed by the same letter (s) are not significantly different according to Duncan's, multiple range test (P=0.05)

Table 2: Evaluation of fruit length, fruit diameter, leaf total carbohydrate and total Chlorophyll for some mandarin cultivars.

Cultivars	Fruit length cm		Fruit diameter Cm		Leaf Total Carbohydrate%		Leaf Total chlorophyll (SPAV unite)	
	2014	2015	2014	2015	2014	2015	2014	2015
Fermont	4.35^{d}	4.73^{c}	4.68^{d}	5.13^{c}	6.93^{b}	8.19^{a}	70.23^{b}	70.70^{ab}
Mucott	4.41^{c}	4.61^{d}	5.2^{c}	5.27^{c}	8.24^{a}	8.3^{a}	72.53^{ab}	74.73^{a}
Balady	4.54^{b}	4.77^{b}	5.70^{b}	5.47^{b}	5.9^{c}	5.78^{b}	71.10^{b}	64.63^{c}
Ponkan	5.25^{a}	4.81^{a}	6.69^{a}	6.35^{a}	5.94^{c}	5.24^{c}	73.6^{a}	66.70^{bc}
L.S.D0.05	0.0458	0.0341	0.186	0.195	0.7361	0.44	2.44	4.12

* Each value represents the mean of three replicates.
** Values within a column followed by the same letter (s) are not significantly different according to Duncan's, multiple range test (P=0.05)

Table 3. Evaluation of VC, Total soluble solids, acidity and TSS/acidity for some mandarin cultivars.

Cultivars	Vitamin C (Vc) mg/100ml juice		Total soluble solids (TSS %)		Acidity (%)		TSS / Acidity	
	2014	2015	2014	2015	2014	2015	2014	2015
Fermont	38.00^{b}	41.33^{b}	11.63^{ab}	10.77^{bc}	0.456^{ab}	0.379^{a}	25.57^{b}	٢4.8^{a}
Mucott	38.67^{b}	41.67^{b}	10.77^{b}	10.40^{c}	0.468^{a}	0.413^{a}	22.99^{c}	25.24^{a}
Balady	42.67^{b}	46.00^{b}	11.33^{ab}	12.40^{a}	0.483^{a}	0.430^{a}	23.47^{c}	28.84^{a}
Ponkan	66.67^{a}	69.00^{a}	12.03a	b11.60^{a}	0.417^{b}	0.412^{a}	28.86^{a}	28.19^{a}
LSD.05	4.9947	5.0169	1.0429	0.8916	0.0406	0.0546	2.0357	4.3266

* Each value represents the mean of three replicates.
** Values within a column followed by the same letter (s) are not significantly different according to Duncan's, multiple range test (P=0.05).

3.4 Mineral Composition

With regard to data in Table (4) Murcott mandarin cultivar had significantly higher leaf nitrogen (2.4%) than Fermont (2.23%) and Chinese (Ponkan) (2.28%) cultivars in 2014. In the meanwhile, Murcott mandarin cultivar in 2014 and Balady mandarin cultivar in 2015 had significantly the highest leaf phosphorus (0.681% &0.628%, respectively) as compared with other all cultivars. Also, Chinese mandarin cultivar had significantly the highest leaf potassium content (1.98 and 2.06%) than all other cultivars. Also, Balady mandarin (1.5&1.81%) contained significantly higher leaf potassium in comparison with Forment (1.27&1.19%) and Murcott (1.39&1.3%). Moreover , (Fermont cultivar tended to have significantly higher leaf magnesium (0.521% and0.497%) as compared with Balady (0.308% and0.375%) and Chinese (0.369% and 0.359%) cultivars in the first season and all other cultivars in the second season .The data in Table (4) also, indicated that Chinese cultivar in 2014contained significantly the highest leaf calcium content (4.43%) and had significantly higher leaf calcium content (4.68%) as compared with Murcott mandarin cultivar (3.34%) in 2015.These results were similar with [42, 43, 44, 45], they found that leaf nitrogen was1.7%-2.81% on Nagpur mandarin,1.97%-2.56% on Khasi mandarin ,2.28%-2.53% on Kinnow mandarin and1.98%-2.57% Mosambi sweet orange and found that leaf Phosphorus 0.09% – 0.15% on Nagpur mandarin, 0.09% – 0.10% on Khasi mandarin 0.11%-0.15% on Kinnow mandarin, 0.91%-0.17% Mosambi sweet orange, also, Found leaf Potassium was 1.02 – 2.59%, on Nagpur' mandarin, 0.99 – 1.93% on Khasi mandarin and 1.34-1.57%on Kinnow mandarin and1.33-1.72% on Mosambi sweet orange. [46, 33] found that leaf nitrogen of Nagpur' mandarin ranged from1.73%-2.37. Also, found that leaf phosphorus on Nagpur' mandarin was 0.095% -0.151and leaf Potassium of Nagpur mandarin ranged from1.49-1.98%. Also, Koo et al 1984 on orange indicated that leaf magnesium optimum level was 0.30 – 0.49 and leaf calcium optimum level was 3.0 – 4.9% on orange and [47] on citrus reported that the Satisfactory Ca (2.5 - 5.5) as the leaf level of calcium is normal.

3.5 Genetic Variation of Mandarin Cultivars based on RAPD-PCR

Random amplified polymorphic DNA (RAPD) molecular marker was used to characterize and determine genetic diversity and phylogenetic relationship (genetic similarity) to four mandarin (*Citrus reticulate*, Blanco) cultivars. RAPD is widely used with whole genomic DNA and random primers to assess genetic diversity among plants. In the present study, the DNA was extracted from four mandarin (*Citrus reticulate*, Blanco) cultivars; Fermont, Murcott, Balady and Chinese (Ponkan) using nine RAPD primers produced 157 fragments. Then it was subjected to the RAPD-PCR technique using nine different arbitrary primers (Table 5). To differentiate between DNA bands or fragments of the four cultivars. Study of the banding patterns showed that primers RAPD2 generated the most

Table 4: Evaluation of leaf nitrogen, phosphorus, potassium, magnesium and calcium for some mandarin cultivars.

Cultivars	N%		P%		K%		MG%		Ca%	
	2014	2015	2014	2015	2014	2015	2014	2015	2014	2015
Fermont	2.23^{c}	2.34^{a}	0.153^{c}	0.218^{b}	1.19^{c}	1.27^{c}	0.521^{a}	0.497^{a}	3.45^{b}	3.67^{ab}
Mucott	2.4^{a}	2.33^{a}	0.681^{a}	0.230^{b}	b1.3^{c}	1.39^{c}	0.511^{a}	0.400^{b}	3.17^{b}	3.34^{b}
Balady	b2.37^{a}	2.35^{a}	0.555^{b}	0.628^{a}	1.57^{b}	1.81^{b}	0.308^{b}	0.375^{b}	3.23^{b}	b3.51^{a}
Ponkan	2.28b^{c}	2.39^{a}	0.174^{c}	0.224^{b}	1.98^{a}	2.06^{a}	0.369^{b}	0.359^{b}	4.43^{a}	4.68^{a}
LSD.05	0.1025	0.1106	0.1047	0.0396	0.2103	0.2392	0.1153	0.089	0.4547	1.217

* Each value represents the mean of three replicates.
** Values within a column followed by the same letter (s) are not significantly different according to Duncan's, multiple range test (P=0.05)

polymorphic bands (92.3%), while the least polymorphic bands (0%) were generated by the primer P1. The DNA banding patterns, with the arbitrary used 9 primers, for the 4 mandarin cultivars under study are shown in Figure (2). RAPD-PCR that used DNA as a template with the nine primers showed constantly different banding patterns with reproducible polymorphic bands that variable in size and number. The highest amplification was with the primer RAPD4 which generated 5 monomorphic bands and 10 polymorphic bands in total 15 with the percentage of polymorphic 66.6% followed by the Es10A26 which generated 11 monomorphic bands and 3 polymorphic band in total 14 RAPD patterns with 21.4% polymorphic as shown in Figure (2) and Table (5), while the primer RAPD6 showed the lowest band monomorphic, it was 3 monomorphic and 3 polymorphic bands in total 6 RAPD patterns. RAPD2 succeeded in producing the largest amplified fragment which was in the range of 1100 bp to 1200 bp as well as, the smallest fragment 200 bp. A total number of fragments used for cluster analysis. The obtained pattern for each species was scord for the presence or absence of each band. The presence or absence band among the species was called a band polymorphic, while monomorphic band if the band was present in all four species. Moreover, Fermont mandarin gave the highest number of amplified fragments for each primer was used in this study. While, Murcott, Balady mandarin and Ponkan resulted in the lowest number of total amplified fragments and same pattern band in different primers were used. Our data agree with those of [48, 49, 50], they reported that RAPD technique has been used for DNA fingerprinting and investigate genetic diversity. This technique was used as a precise strategy to identify *Citrus* species, cultivars, and biotypes. Also, our RAPD products of mandarin samples were identified three specific RAPD markers by [51].

3.5.1 RAPD Phylogenetic Relationships

The phylogenetic tree derived from RAPD patterns with the examined species was presented in Figure (3). The data separated the four mandarin cultivars in two major clusters (cluster A and cluster B). The major cluster A consists of Fermont. The major cluster B consists of two sub-clusters (B1 & B2). Sub-cluster B1 includes two species on same line it is Murcott and Balady. While, sub-cluster B2 contained only one species which is Ponkan. In another study, [52] reported that, all the Mandarin cultivars were grouped together in one cluster. While, Dancy and Bonkan were placed in another cluster because they were very closely related and clearly separated from Clementine and Satsuma. Similarly, using the same processes based on combination between RAPD and SSR, the agree results with the phylogenetic tree cleared two main clusters, the first main cluster contained Citrang C35 and Balady mandarin, while the second main cluster was divided to two sub-clusters, the sub-cluster 1 consisted of Sour orange, Rangpur lime, Volkamer lemon and Macrophylla. while, the sub-cluster 2 included two groups, the group 1 contained Cleopatra mandarin, while the group 2 divided two sub-groups, the sub-group 1 contained Citrang troyer and the subgroup 2 included of Kinnow mandarin, Nour mandarin, Cinoza mandarin, Avanamariana mandarin and Vana mandarin. The sub-group 2 consists two branches the branch 1 contained of Fidel mandarin, Minneola mandarin and Fermont mandarin. Otherwise, the branch 2 included Dancy mandarin, Chinese mandarin and Murcot mandarin.

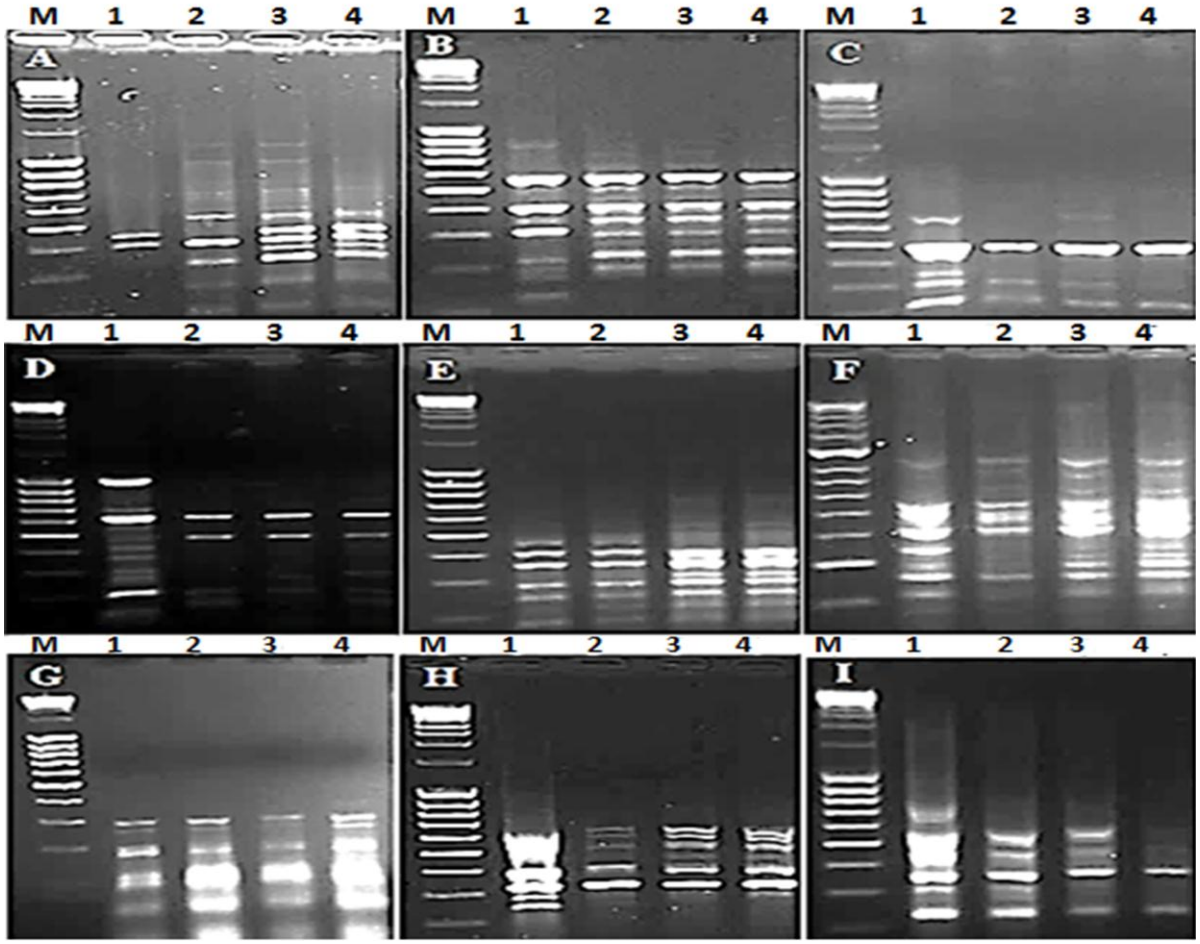

Fig. 2: 2% Agarose gel electrophoresis of RAPD-PCR using primer's (A):RAPD2; (B):RAPD4; (C):RAPD6; (D):RAPD8; (E):RAPD10; (F):Es10A26; (G):P1; (H):S1; (I):S2; M, 1Kbp DNA marker, Lane 1, Fermont; Lane 2, Murcott; Lane 3, Balady; Lane 4, Ponkan, respectively.

Table 5: Sequence of RAPD primers, numbers and fragment, number of polymorphic fragments, Polymorphism percentage; Polymorphic Information Content obtained from 4 cultivars (*Citrus reticulata)* plant selection of Egypt using 9 random primers.

No.	Primers	Sequences 5`-----3`	Total number of bands	Number of polymorphic bands	% polymorphic
1	RAPD2	ATGCCCCTGT	13	12	92.3
2	RAPD4	CCTTGACGCA	15	10	66.6
3	RAPD6	AAAGCTGCGG	6	3	50
4	RAPD8	ACCTGAACGG	13	10	76.9
5	RAPD10	GAGAGCCAAC	12	5	41.6
6	Es10A26	CAGGGGACGA	14	3	21.4
7	P1	CAATGTAACTTAAAGTGCCTA	9	0	0
8	S1	TCCGTAGGTGAACCTGCGG	10	8	80
9	S2	CTTGGTCATTTAGAGGAAGTAA	9	5	55.5
Total	--------	--------	100	55	---

The genetic distance and genetic similarity for 19 Citrus cultivars were showed by phylogenetic analysis [53].

3.6 Detection and Identification of MDH gene, Sequencing and Phylogenetic Analysis

In the present study, the expected band of MDH gene approximately 1000 bp was amplified from mandarin cultivars leaves such as (Fermont, Murcott and Chinese) had been the highest expression level of Malate dehydrogenase gene, while, the MDH gene expression was absent in Balady cultivar and not PCR product was amplified as shown (Figure 4A). The DNA sequences were aligned using the NCBI-Blast. The rooted phylogenetic tree was constructed using the neighbor-joining method with bootstrapping analysis. The MDH gene sequence of Fermont cultivar is closely related to C. reticulata isolate CM33 haplotype hp2 MDH gene (KT175680) with similarity 76% and MDH gene sequence of Murcott and Chinese cultivars are closely related to C. reticulata cultivar Encore mandarin haplotype 1 MDH gene (EU254108) and C. meidentity of 98% (Figure 4B). In the same case, other studies were reported that the malate accumulation in yeast, hairy roots in Arabidopsis and cotton were increased by overexpression of MDH. In contrast, the orthologue of GaMDH13 i.e., the MdcyMDH in apple (accession no. DQ221207), which making ease the transportation of malate into vacuole by generating electrochemical gradient and contributes to cell expansion, whilst suppressor had lower of malate [18].

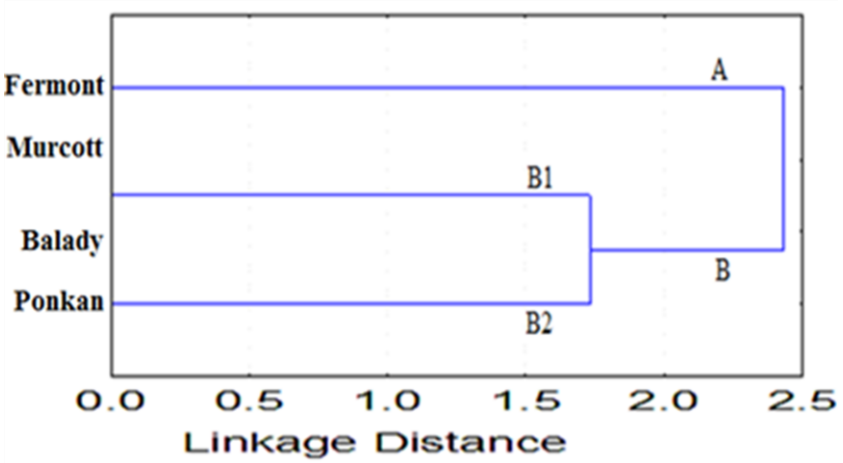

Fig. 3: The phylogenetic tree constructed on the basis on RAPD profile with nine primers using the DNA of the four mandarin cultivars.

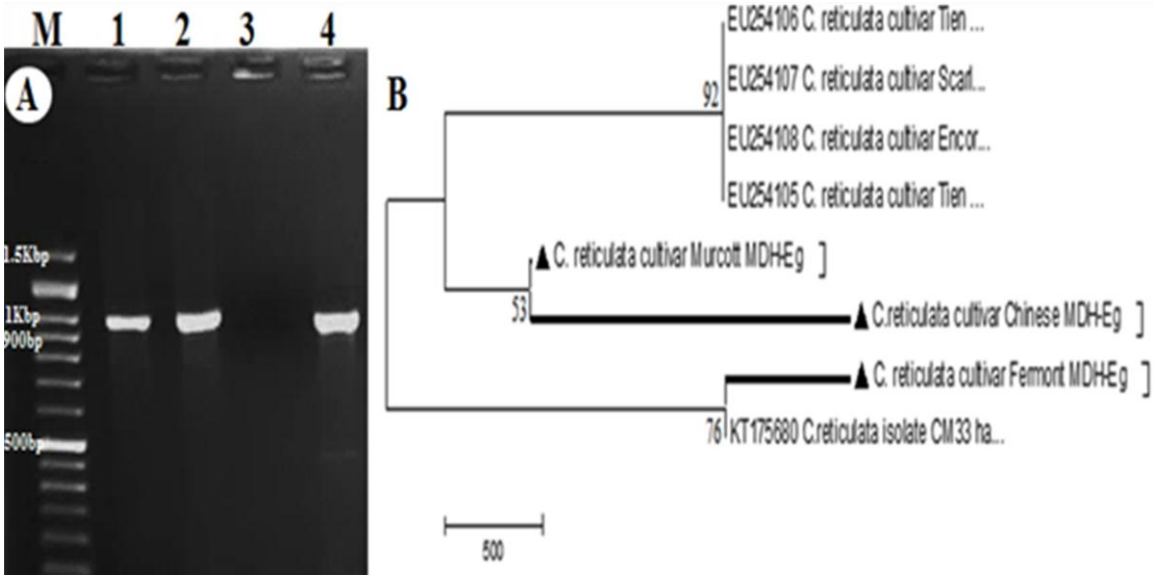

Fig.4: A) 2% Agarose gel electrophoresis of PCR product of MDH gene. M, 1.5 Kbp DNA marker; Lane 1, Fermont; Lane 2, Murcott; Lane 3, Balady; Lane 4, Ponkan. B) Phylogenetic tree relationship of mandarin (*Citrus reticulate*, Blanco) cultivars; Fermont, Murcott and Ponkan for MDH gene.

4 Conclusions

In conclusion, using RAPD markers with whole genomic DNA provided the investigation the chance to match, distinguish Citrus cultivars with specific fragments or alleles and are able to assess genetic diversity among plants. RAPD Operons stated great PIC and GD values and recommended to be used in Citrus cultivars analysis. It can be suggested that the usage of RAPD markers is capable in identification fractional genome in breeding programs. Random primers can differentiate, estimate, characterize and determine genetic diversity and phylogenetic 13 relationship (genetic similarity) among four mandarin Citrus reticulate, Blanco) cultivars, Fremont, Mucott, Balady and Ponkan.

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