International Journal of Chemical Studies

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Evaluation of citrus germplasm for physico-chemical and morphological traits

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DOI: https://doi.org/10.22271/chemi.2021.v9.i1f.11264

Abstract
Citrus fruit tree has great commercial importance with mandarin (*Citrus reticulata)* as the second most important citrus crop and includes a wide range of true and hybrid cultivars; however, there is a lack of studies about the characterization and evaluation of germplasm for Physico-chemical and morphological traits. A study on varietal selection and evaluation has been underway at National Citrus Research Programme since 2005-06. Evaluation of citrus species for various quantitative and qualitative traits related to leaf, fruit and seed morphology at phenotypic and genotypic level using morphological and random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats (ISSR) markers confirmed the existence of a significant variability among all genotypes. The juice content has maximum positive association with pulp weight and then with the fruit weight, equatorial diameter and polar diameter with respect to genotypic and phenotypic level only. TSS and specific gravity was negatively correlated with juice content with respect to genotypic and phenotypic levels. Thus, morphological and molecular markers can be effective tool for successful establishment of genetic correlation among different citrus group. The present review paper will emphasize the evaluation of mandarin germplasm through correlation and path analysis for growth and yield related traits and various Physico-chemical attributes.

Keywords: Citrus, characterization, genotypes, mandarins, morphological traits, molecular marker

Introduction

Citrus is one of the most important fruit crops which belongs to family Rutaceae and it is native to china. The total production of citrus fruits in world is 139.80 million tons from an area of 9.08 million hectare, in which India is in third position in production of citrus followed by China (35.47 million tons) and Brazil (19.07 million tons) (Rana *et al.,* 2020a) [41] in which orange, mandarin, grapefruit and lemon correspond to 98% of the production (Gomes *et al.,* 2020). It includes some of the major species of citrus such as *Citrus aurantifolia* (Lime),*C. limon*(Lemon), *C. maxima* (Pummelo), *C. medica* (Citron), *C. paradisi*(Grapefruit), *C. sinensis* (Sweet orange) and *C. reticulata*(Mandarin).

Among various citrus species, mandarin (*Citrus reticulata*) is leading in terms of area and production followed by sweet oranges which is primarily the Mosambi (Rattan *et al.*, 2020) because of the highest climatic adaptation among the cultivated citrus (Koehler *et al.,* 2003) [28]. Mandarin group is comprised of numerous species as well as intergeneric and interspecific hybrids which made them the most phenotypically heterogeneous in the genus Citrus. Mandarin (*C. reticulata*) together with the grapefruit (*C. maxima*) and citron (*C. medica*) are three basic species of subgenus Citrus (Pal *et al.,* 2013) [38]. The classification of mandarins is still controversial. Swingle (1967) [65] categorized all mandarins in threetaxa (*C. reticulata, C. tachibana and C. indica*), the majority being in *C. reticulata* while Tanaka (1977) [66] distinguished 36 mandarin species. Kinnow (*Citrus nobilis* × *Citrus deliciosa*) is a hybrid variety of the mandarin group of citrus fruits (Kumar *et al.*, 2018) [29]. Mandarins are mainly consumed as fresh fruits; they are source of vitamins and fiber and also contain secondary metabolites including antioxidants like ascorbic acid, phenolic compounds, flavonoids and limonoids- important for human nutrition and health (Jayaprakasha and Patil, 2007) [23].

Exploitation of genetic potential through crop improvement

The advancement in breeding for the economically important characters which are mostly environmentally influenced is examined by the magnitude as well as nature of their genetic

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variability (Gandhi *et al.*, 2001; Rana *et al.*, 2020b) [40]. Further, the available genetic potential of crops enable its proper exploitation for crop improvement, crop production practices like intercropping (Singh *et al.*, 2014; Singh *et al.*, 2016a,b,c,d) [54, 55], secondary metabolites production, shortening of juvenile cycle in perennial species, development of disease resistance etc. These constituents may differ with the cultivar, stage of maturity and season (Singh *et al.*, 2017) [52]. Thus, significance of germplasm has also been confirmed by Singh *et al.* (2018a) [58] in addition to cultural practices *viz.*, intercropping and nutrient management practices in different fruit species (Singh *et al.*, 2019) [49] as it may be responsible for variation in biological efficiency as food producers and the rate of dry matter production per unit area per day among all crops by efficient solar energy transfer (ESET) (Lallawmkima *et al.*, 2018a,b; Singh *et al.*, 2016a; Singh *et al.*, 2018b,c) [32, 55, 60]. Evaluation and induction of resistance against biotic and abiotic stresses has manifold advantages in crop improvement. The improvement of germplasm sustains the production under unfavourable conditions and enables the plant to nullify the impact of adverse environments (Mal *et al.*, 2018) [34]. In order to attain this objective, incorporation of abiotic and biotic strain lenience genes along with other genes that increase genetic yield potential of varieties is required (Singh *et al.*, 2018d) [61]. Shortening of the juvenile period can reduce the selection cycle, thereby decreasing the associated costs of breeding programs. Tree fruit breeders have developed many methods to accelerate flowering to obtain an earlier assessment of new seedlings through exploitation of genetic potential (Anmol and Singh, 2018) [7]. Thus, it is necessary to evaluate the performance of available genotypes for vegetative growth and yield affecting traits like runner production in strawberry (Wineet *et al.*, 2020). Heterozygosity is common feature of citrus which provides adequate genetic recourses for the crop improvement programme (Kamatyanatti *et al.*, 2018) [25]. Traditionally, morphological characters have been used to identify and characterize a species (Sharma *et al.*, 2016) [53]. However, there is a high level of genetic variability which can sometimes make an accurate separation for each variety. Although, there is a large amount of variability within the Citrus genus with which the breeder can work and closely related genera provide a wider selection of characters (Pal *et al.*, 2013) [38].

In modern crop improvement programmes, a number of molecular approaches have been potentially identified and applied for explaining the genetic diversity in crop. However, the morphological characterization is still occupying significant importance for identification of potential cultivars in horticultural crops as proposed by Susandarini *et al.* (2013) [64] and is being used as the primary step for identification and assessment of cultivars and genetic diversity (Rodríguez *et al.*, 2009; Elameen *et al.*, 2011) [43, 16]. Characterization of germplasm is essential to provide information on the traits of accessions assuring the maximum utilization of the germplasm collection to the final users. The recording and compilation of data on the important characteristics which distinguish accessions within a species, enables an easy and quick discrimination among phenotypes. It allows simple grouping of accessions, development of core collections, identification of gaps and retrieval of valuable germplasm for breeding programmes, resulting in better insight about the composition of the collection and its genetic diversity. It also facilitates a check on the trueness to-type of homogeneous samples, allowing detection of misidentifications or duplicates and indicating possible errors. Usually the maintenance and evaluation of germplasm is based on phenotypic features such as morphological, physiological or horticultural descriptions (Deshmukh *et al.,* 2015) [14].

Morphological characterization in combination with molecular markers would be more rewarding in terms of accurate identification and characterization of most closely related cultivars at intra-specific level. Presently, molecular marker techniques are routinely used for proper characterization, management and conservation of germplasm collections of horticultural species (Karp, 1997) [26]. Several molecular markers *viz.* random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), simple sequence repeats (SSRs), amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), coding and non-coding regions of chloroplast DNA, internal transcribed spacer (ITS) region, etc. can be used for analysis of genetic diversity and relationships, cultivars identification, linkage mapping, and molecular phylogeny of Citrus (Corazza *et al.,* 2002; Gulsen and Roose, 2001; Jena *et al.,* 2009; Pang *et al.,* 2007) [12, 20, 24, 39]. Among the molecular markers, RAPD and ISSR markers were extensively used to study genetic diversity and relationships in Citrus species (Capparelli *et al.,* 2004; Das *et al.,* 2004; Kumar *et al.,* 2010; Shahsavar *et al.,* 2007; Siragusa *et al.,* 2006) [10, 13, 30, 46, 62]. Strawberry cultivars have wider diversity enabling the cultivation under temperate, subtropical and tropical condition (Anmol and Singh, 2019a) [5]. A number of studies have been carried to understand genetic makeup and inheritance pattern of various traits, some of the techniques are: studies of linkage map through use of molecular marker like RAPD (Random Amplified Polymorphic DNA), AFLP (Amplified Fragment Length Polymorphism), SCAR (Sequence Characterized Amplified Region) etc; QTL (Quantitative Trait Loci) studies and marker assisted selection; and microsatellite linkage map. The various sex expression related studies had confirmed the existence of hermaphroditism in diploids and trioecious conditions in polyploids (Anmol and Singh, 2019b) [6]. The fruit characterization is very important to determine the genetic variability of a species that can support breeding programs, as well as its relationship with environmental factors (Nascimento *et al.,* 2014) [37]. The knowledge of genetic diversity among accessions is very important to support breeding programs because it allows to characterize germplasm, control genetic erosion and registry new cultivars, and through that information breeding material can be selected (Youseif *et al.,* 2014) [67].

Morphological characterization and application of molecular markers

Koehler *et al.* (2003) [28] characterized 37 mandarin (*Citrus* spp.) genotypes from south-Brazil through integration of morphological approach on various agronomic traits related to leaves, flowers and fruits, and study based on microsatellite markers. It was suggested that all genotypes are suitable for commercial cultivation while the Satsuma variety was recommended for breeding programs. The dendrogram constructed with the morphological results divided the 37 cultivars into four groups, while that obtained with the microsatellites clustered 35 of the 37 cultivars into three groups only.

Akhter *et al.* (2009) [3] were used fourteen accessions of Jamir (*Citrus jambhiri*) to observe the variability among them by RAPD markers. The highest genetic distance value 0.7444

was observed between the accession CJ04 and CJ10 and between CJ10 and CJ12. On the other hand, the lowest distance value 0.1193 was found among the accession CJ15 which revealed that there is a great genetic difference among the accessions. Hence, RAPD offer a potentially simple, rapid and reliable method to evaluate the variability among all species of plant. Similarly, Baig et al. (2009) [9] used Random Amplified Polymorphic DNA (RAPD) markers to evaluate genetic similarity and inter-relationship among 18 citrus genotypes. Out of 40 decamer primers screened, 25 were selected which produced 250 markers; of which 231 were polymorphic and some species or cultivar specific RAPD markers. They confirmed high degree of genetic variability among genotypes with variable origins and Jatti-Khatti and King Mandarin were reported as genetically most diverse species. However, Zerihun et al. (2009) [69] were used the twenty-five SSR primers to assess polymorphism in twelve genotypes which belong to four species and one hybrid of citrus. The results revealed by the two- and three-dimensional principle component analysis further insured these groupings. The similarity matrices ranged from 0.439 (Cleopatra and Blood Red) to 0.801 (Baramasi and Badri lemon), with an average of 0.582 across all the genotypes. Some markers produced unique alleles in specific genotypes which could be used to discriminate them from the other.

Because of high heterozygosity and cross fertility, the mandarins show wide regional variation. Kundan et al. (2010) [31] evaluated the fruits of Sikkim mandarin and reported the regional variations with larger and heavier fruits and more seed count fruit[-1] in the eastern and northern districts. The fruits with thin peel, high firmness and juice (%) were identified in northern district; the maximum TSS (12.0 °Brix), reducing sugar (6.20%) and total sugar (9.49%) were identified in eastern district; the high peel thickness, poor firmness, least acidity (1.34%) and maximum TSS/acid (7.98) and sugar/acid ratio (5.63) was reported in western district; while high acidity, minimum TSS/acid ratio (4.71) and sugar/acid ratio (3.05) was noticed in the fruits of southern district. High sensory value was reported in the fruits from western and eastern district with high flavour, sweetness and overall impression of juice. Dorji and Yapwattanaphun (2011) [15] evaluated mandarin genotypes of six districts of Bhutan and confirmed the existence of wide variation in morphological traits with the promising genotypes for yield and fruit quality from Dagana, for highest TSS and lowest seed counts in fruits from Trongsa and for extended harvesting period from Samtse and Tsirang.

Singh et al. (2010) [51] evaluated around 45 morphological traits of six rangpur lime strains at Punjab Agricultural University, Ludhiana campus and reported the maximum variability (CV= 32.18) for seed count[-1], while minimum (CV = 3.2) was reported for leaf length. The genotype Limonaria Rugosoda had the maximum fruit weight (358.3 g), highest TSS (8.0 °Brix) while minimum length (76.6 g) lowest TSS (6.4°Brix) was recorded for Texas rangpur lime. Zandkarimi et al. (2011) [68] studied some physical and chemical characteristics of lemons and lime fruits from 19 genotypes. The evaluation of several characters showed a significant difference between lemons and limes such as the ratio of length to width, TSS/TA, fruit index shape, pH of fruit, fruit juice and phenolic contents. On the other hand, evaluation of morphological traits can be used to identify superior genotypes of each region, which were adapted to the condition of those regions and can be widely cultivated by farmers.

Singh et al. (2011) [48] were used the 25 simple sequence repeat markers to detect molecular polymorphism among 30 citrus genotypes representing seedling selections, clonal selections, exotic and indigenous species. Results confirmed that seedling origin pummelo (G-1 to G-5) are not true pummelo possibly because of their hybrid origin. However, seedless clone (G-18) and early bearing clone (G-20) of Nagpur mandarin showed similarity (78% and 69%, respectively) with Nagpur mandarin. The dwarf cluster bearing probable citrus hybrid exhibited affinity with sweet lime and constituted a group with 54% similarity. However, Jawandha et al. (2012) [22] assess the genetic variability in baramasi lemon, the fruit samples collected and analysed for various physic-chemical attributes. A wide range of variability with respect to fruit attributes like fruit weight, rind thickness, vitamin C content and number of segments had been recorded. This variability may possibly be exploited for the selection of superior genotypes for conservation, evaluation, utilization and a source for crop improvement in future breeding programme under sub-tropical conditions.

Gaikwad et al. (2015) [17] characterized the physical, morphological and biochemical features of 30 pummelo genotypes and reported variations for growth and plant morphology traits including the biochemical parameters like highest vitamin C (40mg/100g) in CG-9, the greatest pH in CG-14 (4.15), the maximum total soluble solids and titratable acidity in CG-29 (10.8 °B and 3.7%). CG-29 recorded the highest titratable acidity of in contrast to 1.08% for CG-23. Ahmed et al. (2015) had also estimated the genetic diversity and phylogenetic relationships among four grapefruit (Citrus paradise), five pummelo (Citrus maxima) genotypes through Inter-simple sequence repeats (ISSR) markers and had reported 29-83% (average 54,9%) of polymorphism by using 13 ISSR primers.

Barbhuiya et al. (2016) [9] assess the genetical structure and diversity of citron (Citrus medica) in wild and domesticated populations. They analyzed 219 individuals of C. medica collected from four wild and eight domesticated populations using microsatellite markers. The results concluded that the domesticated populations showed close genetic relationships as compared to wild populations and pairwise Nei's genetic distance ranged from 0.062 to 2.091 among wild and domesticated populations. Moasosang and Jahangir (2016) concluded that the association of characters revealed that selection pressure on the basis of higher leaf length will improve quality by decreasing peel thickness, increase the number of segments of fruit, juice percent. Similarly, selection for higher calyx diameter will decrease seed number, acidity, higher style length increases fruit weight, size and juice weight but decreases TSS. Selection of lower stamen length is required for lowering peel thickness and increasing TSS.

Hoque et al. (2017) [21] observed the substantial variation for growth, yield and fruit quality in the Lime genotypes. The genotype CA Nar-001 was identified due to highest plant height (2.45 m), base girth (0.13 m) canopy spreading (1.78 m) and fruit size, further it was found to free from disease whereas the other lines suffered from canker. Mallick et al. (2017) studied that the kinnow mandarin is a highly priced commercial citrus fruit for domestic as well as overseas markets. Excessive numbers of seeds in the fruit makes it less preferred mandarin for dessert as well as processing purposes. Attempts were made in the past to collect superior less seeded clones of Kinnow. Besides, efforts were also made to induce variation in Kinnow using physical (γ-ray) and chemical

(ethyl methane sulphonate) mutagens. The non-availability of SSR markers in Kinnow made us look into cross genera marker polymorphism and characterization of these clones. Clustering pattern showed that all the mutants and clones distinctly grouped and were different from the parent. It showed that mutants and clones had distinct molecular changes in them.

Gaikwad *et al.* (2018) [18] were analysed the thirty citrus rootstock genotypes representing four species (*C. jambhiri*, *C. limonia*, *C. pseudolimon* and *C. macrophylla*) using 79 morphological characters. The analysis of variance for the thirty-nine quantitative traits revealed statistically significant differences for all the characters studied among tested genotypes. The unique morphological markers were identified for particularly five rough lemon, two rangpur lime and an alemow genotype, respectively which can be used as diagnostic plant characters such as obloid fruit shape (AKLRLe 18/2/4), ellipsoid fruit shape (AKLRLe 60/10), attenuate leaf apex (AKLRLe 62/A1), mandarin-like leaflet odour and seedless fruits (AKLRLe 62/U1), sinuate leaf lamina margin and truncate fruit base (AKLRLe 62/U2), greenish albedo colour (AKLRLi 18/7/5), spineless (AKLRLi 18/7/7) dentate leaf margin, presence of petiole wing and round cross section shape of axis (Alemow 1).

Akhter *et al.* (2019) [2] evaluated Physico-chemical characteristics of 20 selected citrus fruits germplasm. There was significant variation among the germplasms in relation to fruit characteristics and organolaptic evaluation. Better performance was found in germplasm No. 20 in respect of total fruit weight, weight of seed and skin thickness of fruits. Germplasm No.1 showed better performance in respect of percentage of edible portion and germplasm No. 6 in respect of percentage of non-edible portion. The total soluble solids found higher in germplasm No. 20 (12.23%) and titratable acidity in germplasm No. 16 (49.33%). Vitamin C and carotenoids found maximum in germplasm No. 20 (442.70 mg/100g). Germplasm No. 4 and 12 was better in respect of anthocyanin (0.10 mg/100gm) and flavonoids (0.19 gm) content of fruit pulp. Considering desired fruit characteristics germplasam No. 20 (pummelo) was found better.

Kaur *et al.* (2019) [27] investigated the morphological characterization of 46 accessions collected from different parts of India on the basis of 42 phenotypic characters including leaf, fruit and seed characters. Pair-wise similarity analysis revealed moderate to significant variation in the 46 accessions of pummelo accessions.

The results derived from this study indicated significant level of variability present within the pummelo group in India, which can be used as important source of genetic diversity and for future breeding programmes. Sayed *et al.* (2019) [45] investigated the morphological characterization, pollen grain fertility and sterility as well as fruit chemical characterization (vitamin C, PH, TSS and acidity) of 13 mandarin varieties (Citrus spp.). The best total soluble solids were determined in the Abd El-Razik (15.1%), whereas the least ones were found in Satsuma (9.13%). Chine mandarin recorded the highest titratable acidity of 3.88% in contrast to 0.67% for Sonbol. Studies of pollen grain fertility showed that Cleopatra and Sayed Marri varieties gave the highest pollen fertility (99.84% and 99.44% respectively). Contrarily, the highest pollen sterility was found in Clementine variety (8.41%).

Rohini *et al.* (2020) [44] were characterized the thirty-eight accessions of *C. jambhiri* using morphological and SSR markers for diversity analysis and population structure studies. Population genetic analysis by SSR markers revealed that accessions collected from North Eastern India were most diverse in terms of genetic diversity parameters and genetic distance analysis of populations showed that the accessions from North East and Himachal Pradesh were most similar genetically while population collected from Himachal Pradesh and Karnataka were the most distinct genetically. Stitou *et al.* (2020) [63] characterized the new mandarin hybrids by morphological and molecular analysis. The morphological characteristics suggested that almost all hybrids showed difference statistically highly significant ($P < 0.01$) for leaf length, leaf width, and ratio leaf lamina length/width. The results of cytometry analysis exhibited that all the hybrids were diploid. This study provided a microsatellite-based approach for parentage assignment in hybrids and that will be useful for investigation of genetic background and selection of new citrus species and varieties.

Singh *et al.* (2020) [50] had reported that PAU Kinnow-1 had reflected a genetic divergence from its parent (Kinnow) for leaf related traits which might be due to multiple variations occurred during development of PAU Kinnow-1 through gamma irradiation treatment of Kinnow mandarin. Similar findings for rest of the genotypes like Kara, King, Wilking, and Willow Leaf was also confirmed. Aman (2020) [4] conducted an experiment for floral and fruit characteristics on three lime (Citrus aurantifolia L.) genotypes (Fato Brno 1, Fato Brno 2 and Saeed). They were compared to the local Mexican lime (a standard commercial cultivar) grown in the same orchard. The results showed a wide range of variation in all of the studied characters. The study indicated that Fato Brno 1 and 2 are seedless genotypes, whereas Saeed has a high number of seeds per fruit. Detected variations can be utilized in a breeding programme for Citrus improvement.

Conclusion

Thus, there are several variations in the phenotypes, genotypes or morphological traits among the all citrus species and several molecular markers *viz*. Random Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeat (ISSR), Simple Sequence Repeats (SSRs), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Coding and non-coding regions of chloroplast DNA, Internal Transcribed Spacer (ITS) region, etc. can be used for analysis of genetic diversity and relationships, cultivars identification, linkage mapping and molecular phylogeny of Citrus. Among the molecular markers, RAPD and ISSR markers were extensively used to study genetic diversity and relationships in Citrus species. But due to lack of selection of any superior germplasm, the farmers are planting trees of seedling origin of unknown yield potential and qualityso DNA analyses seems to be compulsory.

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