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Determination of morphological diversity for seed and seedling characteristics in citrus rootstocks

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Abstract
Citrus is an important fruit crop worldwide for its commercial and medicinal values. The successful cultivation of the citrus is depends on potentiality of the rootstock. In the present study, six rootstocks were morphologically characterized to assess the diversity at seeds and seedling stage. Among the rootstocks analyzed, CRH-12 is found better overall performance regarding to the seed parameters. Regarding to the seedlings and leaf characters, NRC-03 and Trifoliate type rootstocks were found better in unifoliate and trifoliate group respectively. The PCA analysis for morphological parameters of rootstocks revealed grouping of rootstocks according to the types. The clustering analysis based on morphological parameters of citrus rootstocks grouped them into two major groups according to seed and seedling characters. The Rangpur lime and Volkamericana is categorized in one grouped whereas CRH-12, CRH-47, Trifoliate orange and NRC-03 grouped in another group. The study can be undertaken for evaluating variation and association among citrus rootstocks which could provide beneficial information for further crop improvement programs in citrus at initial stage.

Keywords: Citrus rootstocks, morphology, seedling, PCA, clustering

Introduction

Citrus fruit is known globally for its commercial and medicinal value. The importance of citrus is due to the fact that it is the fancy fruit which can be grown in diverse climatic conditions from temperate to tropical and arid regions of the world. The major citrus growing states in the country are Maharashtra, Andra Pradesh, Telangana, Madhya Pradesh, Punjab, Bihar, Gujarat and Rajasthan. Rajasthan has the reputation of growing the best quality citrus fruits in India (Anonymous, 2015). Citrus fruit has been cultivating in an ever-widening area since ancient times. The genus Citrus belongs to the subtribe Citrineae, the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family (Webber, 1967) [34]. The most recent reports of citrus research indicated that citrus is originated in the Himalayan region (Wu *et al*., 2018) [40]. But the earlier reports considered that it was originated from the part of Southeast Asia bordered by Northeast India, Burma (Myanmar) and the Yunnan province of China, (Webber, 1967; Scora, 1975; Gmitter and Xulan, 1990; Anonymous, 2010) [34, 30, 18]. The three predominantly known ancestral citrus taxa are citron (*C. medica*), pomelo (*C. maxima*), and mandarin (*C. reticulata*) [Wu *et al.,* 2018] [40]. These taxa interbreed freely, despite being quite genetically distinct, having arisen through allopatric speciation, with citrus species and genera (Curk *et al.,* 2014) [12].

The environmental stresses are badly reduced 30 to 60 per cent citrus production and productivity as well as fruit quality. The main flaws of these obstacles are due to lack of suitable rootstocks in Citriculture (Bar-Joseph and Dawson 2008; Rocha-Pena *et al.* 1995; Byadgi and Ahlawat 1995; Ghosh *et al.* 2018; Lakshmi *et al.* 2014; USDA, 2014) [4, 28, 7, 17, 23]. Various numbers of rootstock are being used at different part of the world with particular interest of citrus. Nevertheless, no single rootstocks have showed the potential against the most affecting stresses (Chadha and Singh, 1990; Castle *et al*., 1993) [11, 10]. The huge variability in citrus at genera and species level, and other citrus relatives can be plays a pivotal role to boost up the citrus production under limited resources and adverse environmental conditions. Despite this vast cultivation, phylogeny and taxonomy of many citrus rootstocks is remaining uncertain. Many molecular techniques have been developed in studying the genetic diversity, (Susandarini *et al.,* 2013) [33] emphasized the practical importance of morphological characters in horticultural plant species as well as in plant systematic for cultivars identification.

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At present, the morphological study is still considered important and has been deployed as an initial step for cultivar identification and diversity assessment at field level (Elameen *et al.,* 2010; Rodriguez *et al.,* 2009) [15, 29]. The recording and compilation of information on the important characteristics which distinguish accessions within a species, enables an easy and quick discrimination among phenotypes. Generally the evaluation and maintenance of germplasm is based on phenotypic features such as morphological, physiological or horticultural descriptions (Diwan *et al.,* 2014) [13]. The traits included in morphological markers are plant height, disease response, photoperiod, sensitivity, shape/ colour of fruits, flowers, seeds, leaves etc. Therefore, the present study was undertaken to evaluate the extent of variation and association among citrus rootstocks which could provide beneficial information for further crop improvement programs in citrus at initial stage.

Material and Methods
Seeds of six citrus rootstocks were obtained from ICAR-Central Citrus Research Institute, Nagpur and Centre of Excellence for Fruits, Sirsa, Haryana. Hundred seeds of each rootstock were sown individually in single cell of portrays and transplanted into polythene bag (45x30 cm) in nursery at ICAR-Central Institute for Arid Horticulture, Bikaner, during 2017-2018. The seedlings were maintained under net-house with regular watering and fertilization. The weight of individual seed and 1000 seeds (test weight) were taken by digital balance (Denver Instruments, New York), and seed diameters and thickness were measured by digital Vernier Caliper. The germination of seeds was observed till the complete germination and the percentage of seed germination was calculated manually. For the polyembryonic observations, the young seedlings were pulled out gently without disturbing the attached germinated seeds. The seeds given more than one seedlings were considered as polyembryonic seedling and the percentage of polyembryony was calculated manually. The survivability percentage of seedling were counted at the time of transplanting the seedling and expressed as (no. of transplantable seedling/total no. of germinated seeds) x 100. The off type (albinism) seedling were counted based on the colour variation i.e. white colour phenotype of the seedling till 2-3 weeks after germination of the seeds. Ten saplings were used to measure the plant height, stem diameter, number of branches, number of internodes and Internodal length and leaf area by using manual measurements. Further the morphological characteristics of leaf used to characterize and describe the rootstock genotypes were followed based on the descriptor prescribed by the International Plant Genetic Resource Institute (IPGR) (Anonymous, 1999) [2], Rome; and Citrus resources (Citrus ID), USA (Amanda and Trice, 2011) [1]. The statistical analysis was done using Microsoft excel. The Principal Component Analysis (PCA) and value based heat map was generated using Clust Vis software (Tauno and Jaak, 2015) [24].

Result and Discussion
Seed and seed germination parameters of citrus rootstocks
Seed quality parameters were significantly varied in different citrus rootstocks analyzed in the present study (Table 1). The seed boldness parameters were highest in CRH-12 i.e. test weight (253.8 mg) seed width (8.03 mm), seed diameters (88.01 mm), whereas it was observed minimum in CRH-47 regards to test weight (59.0 mg) and thickness (2.86 mm), and seed width (4.61 mm) was in Rangpur lime. The earliest germination at 38 days after sowing (DAS) with maximum germination per cent (85.6 %) were observed in NRC-03, whereas maximum days for germination (43.25 DAS) and minimum germination percent (42.93%) were observed in trifoliate orange and Volkamericana, respectively. The survivability of seedling after germination was observed maximum in Volkamericana (83.5%) and minimum in Rangpur lime (55.47%). The maximum albinism was recorded in Rangpur lime (51.06%) followed by CRH-47 (31.21%) and NRC-03 (26.9%). Whereas, the minimum albinism was recorded in CRH-12 (17.36%) and Volkamericana (20.53%). The strong polyembryony behavior was showed by trifoliate orange (41.66%) and NRC-03 (40.84%) but non-significantly however, it was the poorest in CRH-12 (6.05%). In present study, we speculated that seed weight and diameter may be correlated with polyembryony, albinism and survivability of the seedlings. It also observed that seed germination and survivability depend on the kind of rootstocks, seed quality, seed moisture, growing media and environmental conditions. The higher seed weight and diameter of seed were having higher polyembryony and survivability as well as lower albinism. The possible explanation because of higher weight and size of seed possessed more portion of endosperm nourished the growing seedlings (Bewley and Black 1994; Bowman, *et al.,* 1995; Ohta and Ishibashi, 1957) [5, 6, 25]. Similarly, polyembryony conditions also vary with type of rootstocks (Atabekova, 1957) [3], mode of pollination and age of tree (Furusato *et al.,* 1957; Filho *et al.,* 2002) [3], environment and growing conditions (Carvalho and Silva 2013) [9]. It was also reported that polyembryonic seedlings shown better tolerance to salinity stress (Gora *et al.,* 2017; 2018) [19, 18]. According to Frost and Soost (1968) [16], polyembryony rate was varied from 13% (cross-pollination) to 73% (selfing-pollination) in citrus rootstocks. Thus, the observations recorded by these groups supported our study.

Table 1: Morphological characteristics of seed and germinations in citrus rootstocks

Rootstocks	Test weight (mg)	Seed length (mm)	Seed width (mm)	Seed diameter (mm)	Seed thickness (mm)	Germination (Days)	Germination (%)	Survivability (%)	Albinism (%)	Polyem-bryony (%)
CRH-12	253.8	10.95	8.03	88.01	5.20	36.25	67.91	80.38	17.36	20.84
Trifoliate orange	83.8	8.46	5.12	43.3	4.24	43.25	81.11	78.1	23.35	41.66
Rangpur lime	67.0	11.56	4.61	53.36	3.58	39.75	58.72	55.47	51.06	19.75
Volkamericana	119.8	13.2	5.45	72.28	4.98	41.50	42.93	83.5	20.53	27.05
CRH-47	59.0	9.16	4.64	42.48	2.86	38.00	62.79	73.6	31.21	6.05
NRC-03	89.8	8.92	5.54	49.39	4.72	33.75	85.6	76.98	26.9	40.84
SEm±	4.76	0.33	0.27	3.62	0.20	0.98	1.17	1.02	1.12	1.35
CD at 5%	14.03	0.98	0.79	10.67	0.59	2.98	3.44	3.01	3.29	3.97

Leaf morphometric analysis of citrus rootstocks

Regarding to leaf morphometric analysis, we observed similar pattern of leaves of citrus rootstocks accordance to IPGR, Rome; and Citrus resources (Citrus ID), USA suggested leaf morphological parameters (Table 2 and Fig 1). Three rootstocks *viz.* Rangpur lime, Volkamericana and NRC-03 showed unifoliate type of leaves whereas CRH-12, CRH-47 and trifoliate orange showed trifoliate type leaves. The leaf apex was observed acute type in CRH-12, CRH-47 and Volkamericana and obtuse type in the remaining rootstocks. The leaf margin and lamina shape were similar in all rootstocks except it was serrated type in Rangpur lime. The leaf texture was observed rough in trifoliate type rootstocks whereas it was smooth in unifoliate type of rootstocks.

Table 2: Variability for leaf characters of citrus rootstocks

| Parameters	Leaf type	Leaf apex	Leaf margin	Leaf lamina shape	Leaf texture
CRH-12	Trifoliate	Acute	Entire	Elliptic	Rough
Trifoliate orange	Trifoliate	Obtuse	Entire	Elliptic	Rough
Rangpur lime	Unifoliate	Obtuse	Serrated	Elliptic	Smooth
Volkamericana	Unifoliate	Acute	Entire	Elliptic	Smooth
CRH-47	Trifoliate	Acute	Entire	Elliptic	Rough
NRC-03	Unifoliate	Obtuse	Entire	Elliptic	Smooth
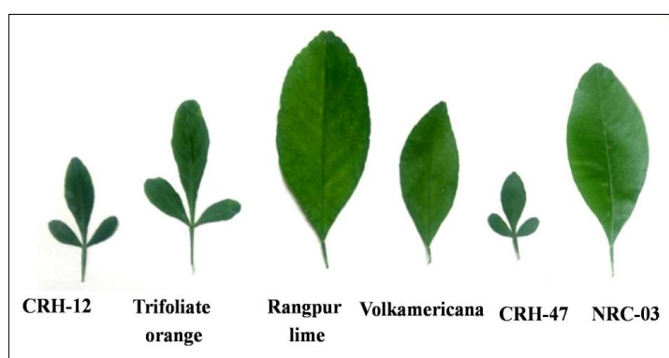
Fig 1: Leaf morphology of citrus rootstocks

Morphometric analysis of nursery seedlings of citrus rootstocks

The morphometric observations of seedlings grown in nursery showed significant variation in rootstocks (Table 3). The seedling vigourness was showed maximum by Volkamericana rootstocks with regards to seedling height (95.4 cm) and, number of internodes (50.6). While the minimum height of seedling (31.2 cm) with comparatively less number of internodes (19.6) were recorded in CRH-12 rootstocks. Further, stem diameter (9.16 mm), internodal length (2.53 cm) and number of branches (11.2) were also observed maximum in Rangpur lime and minimum in CRH-47 rootstocks. Leaf morphology was also significantly varied in analyzed rootstocks. The leaf length (9.42 cm), leaf width (5.12 cm), leaf area (48.2 cm^2) and leaf thickness (0.57 mm) were maximum in NRC-03 while minimum observed in CRH-47 rootstocks. The leaf morphological characteristics also play an important role as classifying citrus species and varieties (Swingle, 1967; Tanaka, 1969; Handa and Oogaki, 1985; Iwata and Ukai, 2002; Camargo Neto *et al.,* 2006 and Du *et al.,* 2007) [34, 35, 21, 22, 8, 14], and pre breeding selection criteria of rootstock breeding. Rough and thick leaf texture with small in size proven leaf longevity, less infected to biotic agents and strict to transpiration checker thus considered more suitable in arid ecosystem (Usman *et al.* 2006; Shokrollah *et al.* 2011; Podda *et al.* 2013) [38, 31]. Besides, it also gives us an idea about their kind of growing habitat as well as their adaptation in different types of environments (Pyakure and Wang, 2013).

Table 3: Morphological analysis of citrus rootstocks seedlings

Parameters	Seedling height (cm)	Stem diameter (mm)	No. of internodes	Internodal length(cm)	No. of branches	Leaf length (cm)	Leaf width (cm)	Leaf area (cm^2)	Leaf thickness (mm)
CRH-12	31.2	4.27	19.6	1.6	4.8	4.18	3.94	16.54	0.41
Trifoliate orange	36.4	4.62	25.2	1.46	3.6	5.04	3.96	19.98	0.5
Rangpur lime	91.6	9.16	37.2	2.53	11.2	8.26	4.26	35.21	0.46
Volkamericana	95.4	8.29	50.6	1.88	9.0	8.98	4.18	37.57	0.5
CRH-47	33.4	4.04	29.6	1.13	0.6	3.74	2.68	10.05	0.4
NRC-03	55.2	5.21	40.4	1.37	6.8	9.42	5.12	48.2	0.57
SEm±	0.89	0.27	1.89	0.09	0.65	0.15	0.10	0.93	0.02
CD at 5%	5.44	0.78	5.57	0.26	1.91	0.44	0.31	2.75	0.04

Clustering analysis of citrus rootstocks based on morphometric parameters

The principle component analysis (PCA) and clustering of data reveals the grouping of organisms and traits based on the similarity among them (Tauno and Jaak, 2015) [24]. Similarly, the citrus rootstocks were classified based on all observed morphometric parameters using PCA and clustering analysis. The PCA analysis revealed the grouping of citrus rootstocks according to their types. For instance, CRH-47 and trifoliate orange were observed as trifoliate type of rootstocks by above studies which were comes into single group in PCA analysis (Fig. 2). Similarly, Volkamericana and NRC-03 were grouped as single component. Contrary, Rangpur lime and CRH-12 were distinct because were grouped separately (Fig 2). Further, all morphometric observations were clustered in term of heat map (Fig 3). The morphometric parameters were clustered into two major groups with sub-groups in heat map. Similarly, the rootstocks were also clustered accordance to their type. The unifoliate Rangpur lime and Volkamericana were clustered in to a single group and CRH-12, CRH-47, NRC-03 and trifoliate orange were clustered into another groups. The NRC-03, though it comes under unifoliate type, however, it contradictorily grouped with trifoliate based on its morphometric characters (Fig 3).
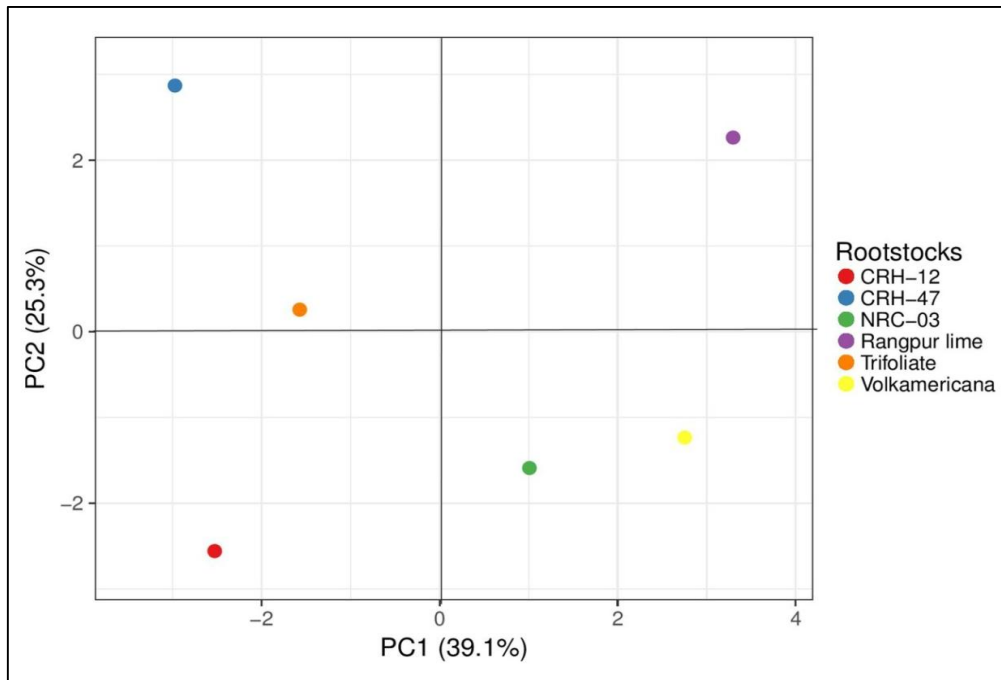
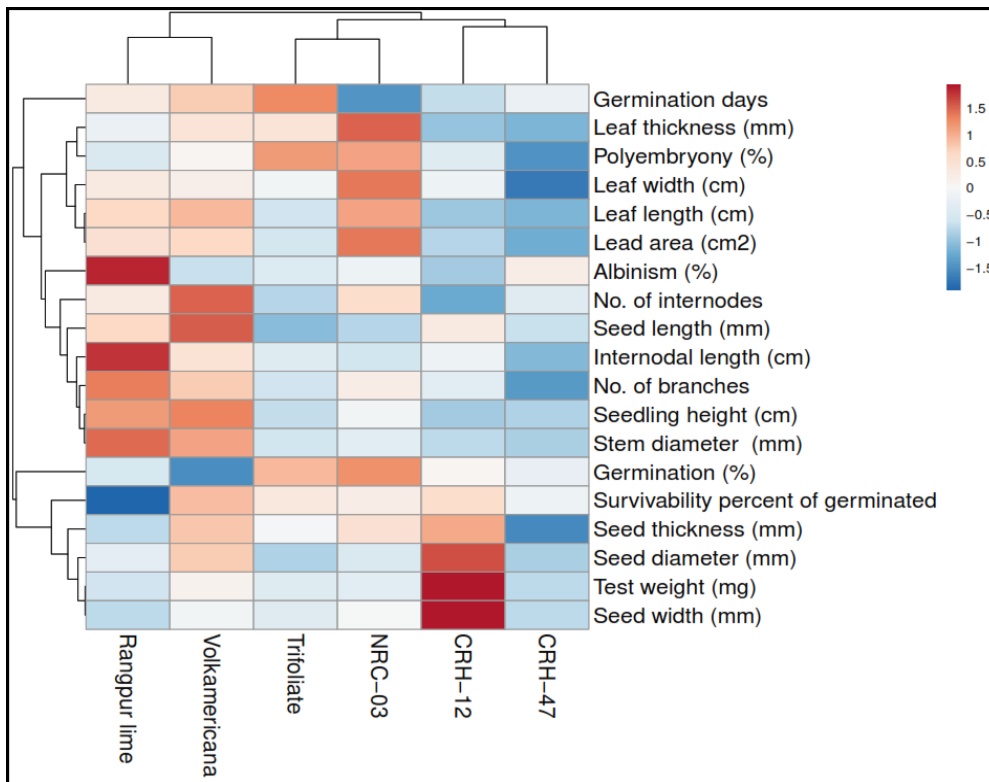**Fig 2:** PCA analysis of citrus rootstocks for seed and seedling characteristics.

Fig 3: Morphological parameters based clustering of citrus rootstocks for seed and seedling characteristics.

Conclusions

It is concluded that NRC-03 rootstocks is better than other unifoliate type rootstocks studied in the present investigation. It showed strong polyembryony behavior with maximum percentage of germination and specific leaf characters pertaining to the economic important traits. Among trifoliate group of rootstocks (CRH-12, CRH-47 and trifoliate orange), trifoliate orange is better in seed and seedling parameters as well as maximum polyembryony behavior that would be helped in uniform and true-to-type nursery stocks production for budding or grafting. The PCA analysis for morphological parameters of rootstocks revealed grouping of rootstocks according to the types. The clustering analysis based on morphological parameters of citrus rootstocks grouped them into two major groups according to seed and seedling characters. The Rangpur lime and Volkamericana is categorized in one grouped whereas CRH-12, CRH-47, Trifoliate orange and NRC-03 grouped into another group.

Acknowledgements

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