Management of citrus genetic resources in India: approaches, applications and achievements

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Abstract

Citrus, representing several wild and cultivated species has its home in Northeast and Northwest India. The occurrence and origin of Indian species was documented by various explorers and horticulturists since 1954 and subsequently refined/modified during the last decade. Besides the commercially cultivated species, several important species like *Citrus indica*, *C. macroptera*, *C. latipes*, *C. megaloxycarpa*, *C. medica*, *C. ichangensis* and *C. assamensis* grow in India in wild and semi-wild state. These wild and semi-wild species of Citrus have immense socio-economic, cultural, religious and medicinal value, besides being important sources for agronomic traits. These genetic resources are important for the citrus industry and crop improvement programmes. The natural diversity of citrus is reportedly shrinking at an alarming rate due to large-scale deforestation and farmers' preference for more remunerable crops. However, only few of the plants of semi-wild species are grown in backyard gardens of tribal farmers in the northeast and north-west India. To achieve comprehensive conservation and to protect these vast genetic resources for utilization, a complementary conservation strategy is to be adopted. The present study concentrated on the documentation of the status of wild, semi-wild and cultivated species of citrus occurring in India, their traditional uses, economic potential and threat perception to aid in designing suitable strategies for their survey, explorations, sustainable utilization, conservation and preservation. The paper also highlights the efforts being made to establish a unique cryopreserved base collection of Indian Citrus germplasm collected from diverse sources, including field genebanks, ensuring long-term conservation.

Keywords: cryobank, embryos, embryonic axes, in situ, on-farm, non-orthodox

INTRODUCTION

Citrus is one of the most economically important fruit crops of India. It is believed to originate in Southeast Asia. India has an enormous diversity of *Citrus* genetic resources, both in cultivated and wild types, comprising 24 species, one subspecies and 78 varieties, including wild, endangered and endemic species (Sharma et al., 2004; Malik et al., 2013). Some of the wild species namely *C. indica*, *C. ichangensis*, *C. latipes*, *C. macroptera*, *C. assamensis*, *C. megaloxycarpa* and *C. medica* have special importance in Indian germplasm being of use in socio-economy and health of local people (Malik et al., 2012a), besides utility in citrus industry and improvement programmes; and therefore need sustainable and inclusive management efforts. Management of *Citrus* genetic resources, some of which are still growing as natural wild and semi-wild populations as recorded from Citrus Gene Sanctuary in Garo Hills (Singh, 1981), would require adoption of complementary conservation strategies. Within the genus *Citrus*, a species-specific conservation strategy is to be developed based on extent of genetic diversity available, mechanism of propagation, reproductive biology of species and present biological status of the species. In situ conservation is, therefore, recommended to conserve the available genetic diversity of such economically important species in the best possible way. For genetic improvement and

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genotype conservation, elite plants are conserved in field genebanks at various horticultural organizations in India. As per the IUCN norms, seven Indian *Citrus* species namely *C. indica*, *C. macroptera*, *C. latipes*, *C. assamensis*, *C. ichangensis*, *C. megaloxycarpa* and *C. rugulosa* are endangered as indicated by threat perception analyses (Singh and Singh, 2003; Malik et al., 2013). Managing genetic resources of *Citrus* where species/genotypes are scattered amongst various stakeholders of National Agriculture Research System (Indian Council of Agricultural Research and State Agriculture/Horticulture Universities), State Horticulture Departments and Ministry of Environment and Forest (custodian of forest and wild flora), is far more challenging. Due to this diverse patronage of national resources, precise documentation of germplasm is presently lacking in *Citrus* and other major indigenous horticultural crops, which need the urgent attention of policy makers. Realizing the importance of management of such resources, present studies were undertaken for exploration, collection, characterization and conservation of Indian *Citrus* germplasm.

MATERIALS AND METHODS

Survey, exploration and collection

For the present studies, several survey and exploration trips were conducted to various states of northeast and northwest India, namely Arunachal Pradesh, Assam, Meghalaya, Mizoram, Nagaland, Sikkim, Tripura, Punjab, Haryana, Himachal Pradesh, Uttar Pradesh, Maharashtra, and Uttarakhand and other parts during 2002 to 2014. Detailed information about indigenous traditional knowledge regarding the use of various plant parts, socio-economic value and livelihood contribution of various important species was collected from the elderly persons of tribal communities and other local inhabitants of the forest and marginal areas. Information about present status of genetic variability, population structure, market value and support system was gathered during the survey of field, local markets and weekly markets. Information on present status of tree count in a population vis-à-vis past status during the last two to three decades was also obtained to assess any changes in the population size of these species. Discussions were held with the tribal farmers, forest officials and other local inhabitants to have their input on the value addition prospects, market support system and policy issues related to conservation and sustainable utilization of this important fruit species.

Cryopreservation and cryobanking

Fruits of *Citrus* and its hybrids were collected from field genebanks, farmer orchards, homestead gardens and natural habitats. The seeds were extracted and processed for embryonic axes (EA) excision as described previously (Malik and Chaudhury, 2006). Embryos and embryonic axes were cryopreserved using methods described earlier based on the species (Malik and Chaudhury, 2006; Malik et al., 2012d).

RESULTS AND DISCUSSION

India has a distinct position in the 'Citrus belt' of the world due to remarkable diversity in *Citrus* genetic resources, both in cultivated and wild species. Apart from the most commonly cultivated species and hybrids, such as citrons, lemons, limes, mandarins, sour oranges, sweet oranges, pummelos and grapefruits, four species, namely Indian wild orange, Khasi papeda, Ichang papeda, and Melanesian papeda were recorded to occur in wild or semi-wild state in Northeast India. Out of 30 *Citrus* species, sub-species and hybrids available in India at least nine are available throughout India, whereas 15 are confined to Northeastern India, nine to South India, six to Northwestern India and one to central India (Malik et al., 2012a).

Exploration and collection

Most of the new *Citrus* species were identified, collected and documented from the northeastern parts of India during the intensive collections taken up by Tanaka (1928, 1937) and Bhattacharya and Dutta (1956). Recent surveys and explorations enumerated loss of

some species and local types from various pockets where these were earlier reported to exist (Singh and Singh, 2003; Malik et al., 2006, 2013). Therefore, there is an urgent need to collect this genetic diversity and document the ethno-botanical as well as indigenous technical knowledge. During the last one and half decades, the National Bureau for Plant Genetic Resources (NBPGR), as institutional priority, undertook extensive explorations and survey programmes for citrus in various parts of India. During these explorations a vast diversity of various *Citrus* species representing important rootstocks, mandarins, acid lime, pummelo, grapefruit and sweet oranges along with the indigenous wild species were collected (Malik et al., 2012a). During these explorations, a major emphasis of NBPGR was on collecting the diversity of wild, semi-wild and endangered *Citrus* species from the entire Northeastern India, for which specific explorations were undertaken from 2002 to 2013. In the last four decades, germplasm representing a sizable diversity of cultivated, wild and rootstocks species and several intermediate *Citrus* types have been collected during various explorations undertaken in India and national identity for more than 1400 accessions has been assigned.

Characterization and evaluation

Indian *Citrus* germplasm, especially that of indigenous origin, has not been sufficiently characterized and evaluated. This is due to more emphasis on use of introduced cultivars and selections from indigenous germplasm. Even if limited germplasm is characterized it has been for phenotypic characters. However, recently molecular characterization of collected and cryostored Indian *Citrus* germplasm was undertaken to assess the existing genetic diversity in the specific groups of *Citrus* (Malik et al., 2012b, c; Pal et al., 2013; Kumar et al., 2012, 2014; Hynniewta et al., 2014). Since citrus is a very diverse fruit crop, detailed evaluation and characterization data would be needed to assess the genetic diversity present in individual species or groups. Indian *Citrus* germplasm has been characterised by several workers on the basis of herbarium observations and morphological descriptions. Chemotaxonomic and numerical taxonomic studies have also aided in the characterization and classification of *Citrus* (Nath and Randhawa, 1969; Singh and Chadha, 1993; Nair and Nayar, 1997).

Field evaluation of Indian *Citrus* germplasm has been undertaken at National Research Centre (NRC) for Citrus, Nagpur and at various other centres under the ICAR-All India Coordinated Research Project (Tropical Fruits-Citrus), where several promising genotypes have been identified in Nagpur mandarin, Khasi mandarin, acid lime, pummelos, sweet oranges and others through multilocation trials. Similarly, production and protection trials including rootstocks performance have been undertaken at various centres for different agronomic traits and diseases (insects, pests and nematodes). At Mahatma Phule Krishi Vidyapeeth, Rahuri, Maharasthra, 140 accessions of different *Citrus* species and allied genera have been evaluated for horticultural performance and resistance to biotic and abiotic stresses (Pujari et al., 1999). At NBPGR various *Citrus* accessions collected during explorations have been characterised for leaf, fruit and seed characters using IPGRI descriptors and also using various molecular markers (Table 1).

Table 1. Genetic diversity studies in *Citrus* species using morphological and molecular markers at NBPGR, India.

Citrus species	Significance	Exploration & collection areas	No. accs. studied	Genetic diversity and relationship studies	
				Morphological markers	Molecular markers
Citrus reticulata (Mandarin)	2nd most important citrus crop worldwide	North, Central, N.E. India	60	11 qualitative, 22 quantitative	ISSR & RAPD (Moderate level of genetic diversity & cultivar specific amplicons)[1]
Citrus macroptera (Melanesian papeda)	Endangered wild sp.	N.E. India	9	10 qualitative, 20 quantitative	RAPD (High level of genetic diversity)[2]
Citrus sinensis (Sweet orange)	Commercially cultivated	Cultivars from Punjab State	89	29 qualitative, 14 quantitative (exhibited high morphological variability)	RAPD (Low level of genetic diversity)[3]
Citrus medica (Indian citron)	Cultivated and wild	Throughout India	46	12 qualitative, 21 quantitative	RAPD & ISSR (Significant diversity among genotypes)[4]

[1]Pal et al., 2013; [2]Malik et al., 2013; [3]Malik et al., 2012c; [4]Kumar et al., 2014.

Conservation

Conservation of genetic resources of a diverse genus like *Citrus* needs appropriate planning and complementarity of conservation approaches. In the present scenario the most appropriate strategy would be to ensure conservation of vulnerable species before they are lost from nature. Ex situ conservation in field genebanks with duplicate collections in cryo genebanks, complemented with in situ conservation in gene sanctuary, national parks and on-farm, especially for wild and semi-wild species of *Citrus* are the best options. Till the appropriate steps and planning for in situ conservation are taken, the field genebank conservation and cryopreservation of seeds, embryo, embryonic axes of germplasm in the cryo genebank are needed to be immediately taken up. However, in view of differences in seed desiccation and freezing tolerances of many *Citrus* species, it necessitates the determination of seed storage behaviour and development of suitable protocols for seed/embryo cryopreservation.

1. In situ conservation.

India established the world's only "Citrus Gene Sanctuary" at Nokrek Biosphere Reserve in Garo Hills of Meghalaya for in situ conservation in 1981, covering about 10,266 ha (Singh, 1981). It is a unique sanctuary, probably first of its kind in the world. It is endowed with highly specified microclimate with a combination of tropical and mild temperate seasons and very high humidity and rainfall. The gene sanctuary is a part of the buffer zone of Nokrek Biosphere Reserve and spreads over the east, west and south Garo hills of Meghalaya. *Citrus* species growing inside the gene sanctuary are still safe, however, the slow rate of regeneration of these species and increasing human intervention around the gene sanctuary area are causes of serious concern. This "Citrus Gene Sanctuary" deserves the status of "Heritage Site" under the provisions existing in "Biological Diversity Act, 2002" of India (Anonymous, 2004). Sharma et al. (2004) and Malik et al. (2013) suggested the creation of more nature reserves, gene sanctuaries and gene parks, and inclusion of *Citrus* species in the social forestry system to safeguard the genetic resources and to allow their

further evolution under the natural stresses. Species-specific conservation strategy should be adopted depending upon biological status, population size, diversity and endemism.

2. On-farm conservation.

India in general and tribal regions in particular still have workable on-farm conservation systems, but in the present context they are also facing threats. In-situ on-farm conservation is a common practice in *Citrus* diversity-rich areas of north-eastern Himalayas. Conservation of *Citrus* genetic resources needs urgent attention so as to protect the existing genetic diversity and to promote cultivation of rare and endangered species that are of great relevance in socio-economical structure of tribal populations of this area. In citrus "local selections" or "farmers' varieties" have been selected or identified since time immemorial in India. Due to their local socio-economic importance and the preference of fruits as a food, in cultural or religious rituals, on-farm conservation for these semi-domesticated, natural selections/cultivars/farmer's varieties have been attempted. These local cultivars are being grown as isolated plants or in small numbers in the homestead gardens, farmers fields and backyards. Such selections need urgent attention for further characterization, evaluation, taxonomical identity and on-farm conservation. In situ on-farm conservation of Kachai lemon in Kachai village of Okhrul district of Manipur is a model of exclusive farmer participation in on-farm conservation and sustainable horticulture providing livelihood support to tribal farmers (Malik et al., 2013). In situ conservation areas for wild and semi-wild species and several local *Citrus* types have been proposed based on a detailed survey in the north-east India. This recent concept and role of custodian farmers in on-farm conservation may be further strengthened, especially since *Citrus* genetic resources of India are highly diverse in nature (Sthapit et al., 2013).

3. Ex situ conservation.

Field genebanks.

More than 20 field genebanks were established in India in National and State institutions, State Agricultural Universities and private nurseries and more than 1,500 accessions are being maintained. In *Citrus* field genebanks maintenance is a challenging task due to strict agronomical and climate requirements and threat of die-back and other *Citrus* diseases. During the last decade a major loss of germplasm from the field genebanks has been noticed due to several biotic and abiotic factors. Due to the need for crop improvement and breeding experiments, all *Citrus* growing nations maintain live plants in the field genebanks and clonal repositories. NRC Citrus, Nagpur maintains the largest collection including 614 accessions of *Citrus* species in central India (Malik et al., 2013). There is an urgent requirement to establish regional field genebanks and clonal repositories for *Citrus* in diversity-rich areas of India for conservation, use and effective exchange of germplasm.

In vitro conservation and cryopreservation of vegetative tissues.

In vitro conservation and cryopreservation has been attempted at NBPGR and on a limited scale at National Botanical Research Institute, Lucknow. In vitro long-term preservation has been attempted in *C. aurantifolia* (Bhat et al., 1992) and in *C. grandis* using shoot apices (Chaturvedi, 2002). In *C. aurantifolia*, root cultures retained their ability to regenerate shoot buds when tested after three years of storage and regenerated plants with a normal diploid chromosome count (Bhat et al., 1992). Similarly, shoot cultures of *C. grandis* were reportedly preserved for more than 32 years (Chaturvedi, 2002). In the case of highly recalcitrant seed species and wild, rare and endangered species like *C. ichangensis*, *C. indica*, *C. macroptera*, *C. megaloxycarpa* and *C. latipes*, in vitro conservation and cryopreservation of shoot tips from in vitro plantlets are being attempted as alternative methods at NBPGR.

Cryopreservation of embryos and embryonic axes.

Storage of *Citrus* germplasm in the form of embryos has an in-built advantage of conserving nucellar embryos, which provides true-to-type plants on recovery. Presently no

reports of seed transmitted viruses in *Citrus* gives extra impetus for conserving germplasm in the form of seeds, embryo and embryonic axes in this genus. Most of the Indian *Citrus* species have been studied for seed storage behaviour and cryopreservation using different explants (Malik et al., 2012d; Normah et al., 2013). Seed storage behaviour of 29 *Citrus* species and related genus *Poncirus*, primarily based on sensitivity of embryo and embryonic axes to desiccation and freezing, have been ascertained. Successful cryopreservation has been achieved using air desiccation-freezing, vitrification and encapsulation-dehydration with varying recovery percentages. However, for cryobanking of germplasm, desiccation followed by fast freezing technique has been used because it is a simple, repeatable and cost effective technique (Malik et al., 2012d). Initial viability of freshly collected embryos and axes was 95 to 100% which progressively declined with duration of desiccation. A 40 h embryo drying led to critical moisture content (MC) of 10%. For axes a critical MC level between 11 to 17% was achieved by 3-5 h drying. In our studies embryos and embryonic axes of *Citrus jambhiri* at 6% and *C. karna* at 8% MC proved to be the most desiccation sensitive with complete viability loss and hence have been characterised as recalcitrant (Malik et al., 2012a; Normah et al., 2013). Across most species the critical MC hydration window for survival after exposure to liquid nitrogen (LN) was slightly wider for the embryonic axes compared with the embryos. At the critical MCs, survival (growth) after cryopreservation ranged from 66 to 82% for embryos and 54 to 96% for embryonic axes. Most of the desiccated seeds of *Citrus* species survived LN exposure well with original germinability percentages. This high recovery rate of genetically stable plantlets with normal growth prompted the establishment of a *Citrus* base collection in the National Cryogenebank at NBPGR, India.

Long-term cryobanking.

Cryopreservation is the only current alternative for long-term conservation of species of *Citrus*, and this method has been extensively attempted using seeds and a wide range of other explants like zygotic embryos, embryonic axes, shoot apices, embryogenic callus, cell suspensions and somatic embryos (Malik et al., 2003; Malik and Chaudhury, 2006). In India embryos and embryonic axes of more than 800 diverse accessions comprising wild and semi-wild species, cultivars, hybrids and some unknown local types were successfully cryobanked at the NBPGR Cryogenebank. 316 accessions of five species were assessed for viability after average storage periods of 6.3 years for *C. reticulata* to 8.4 years for *C. medica*. After this storage, 69 to 81% of the accessions retained ≥70% of the original, post-drying viability (Malik et al., 2012d). Using developed protocols, diverse accessions collected from various parts of India from naturally growing plants and from field genebank collections were successfully cryostored. Seed storage behavior of several wild, semi-wild and lesser known *Citrus* species of India is still to be investigated and germplasm existing as natural wild and in the field genebanks is to be collected and conserved for posterity and sustainable use in the *Citrus* improvement programmes.

CONCLUSION

In conclusion, efficient management of *Citrus* genetic resources in India needs further impetus. Explorations in northeast and northwest India, especially in remote and difficult areas, are to be targeted for collection of rare germplasm. Characterization and evaluation of existing indigenous germplasm using modern tools and techniques need priority to assess the real wealth of available genetic diversity which can be exploited for citrus improvement programmes. As far as conservation of genetic diversity of *Citrus* is concerned, both in situ and ex situ approaches are recommended. In the present scenario most appropriate strategy for *Citrus* germplasm conservation is to adopt immediate ex situ conservation (i.e. field genebanks, clonal repositories and cryobanking) complemented with in situ conservation (in situ on-farm conservation, gene sanctuary and national parks) for wild and semi-wild species.

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