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Dwarfing of Breadfruit (*Artocarpus altilis*) Trees: Opportunities and Challenges

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Authors' contributions

This work was carried out in collaboration between all authors. Authors YZ and SJRU conceived the idea. Author YZ drafted the original manuscript in discussion with author SJRU. Author MBT critically reviewed the manuscript. All authors read and approved the final manuscript.

Review Article

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ABSTRACT

Breadfruit [*Artocarpus altilis* (Parkinson) Fosberg)] is a traditional staple crop grown for its starchy fruit throughout the tropics. It has long been recognized for its potential to alleviate hunger in the region. However, being a tree of 10 – 30m, breadfruit is vulnerable to wind damage. Owing to the continuing trend of global climate change, the success of the species as a sustainable crop for delivering local food security is compromised by the likelihood of more intense tropical windstorms in the island nations. Tree height also forms a major constraint to disease management and fruit harvesting. These imperatives have driven an increasing interest in developing breadfruit varieties with short stature. While a great diversity of breadfruit cultivars with varying nutritional and agronomic characteristics exists, the genetic resource showing dwarfing traits is largely uncharacterised. Historically, there has been no intentional breeding for breadfruit cultivars. The long growth cycle, predominantly vegetative propagation and lack of genome information create challenge for crop improvement through traditional breeding. In this review, we highlight the current knowledge of plant dwarfism and its application in agricultural practices and genetic improvement for dwarf phenotype, and present options and tools for breadfruit dwarfing with special reference to natural genetic variability for dwarfing rootstocks, plant growth regulators, potential of mutagenesis and its combination with the currently

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established *in vitro* propagation protocol in breadfruit. The role of genetic transformation, high-throughput mutant detection by using Targeting-Induced Local Lesions IN Genomes (TILLING) and tools of next generation sequencing is also discussed.

Keywords: Dwarfism; *Artocarpus*; breadfruit; genetic variability; plant growth regulator; mutagenesis, mutation breeding, genetic transformation.

1. INTRODUCTION

Breadfruit [*Artocarpus altilis* (Parkinson) Fosberg)] is a staple tree crop in the Oceania and throughout the tropics [1]. The millennia of selective breeding by the indigenous peoples of Oceania has resulted in great diversity in morphological, agronomic, and nutritional characteristics among cultivars [2], resulting in hundreds of cultivars [3], some of which have been globally distributed including Central and South America, Africa, India, Southeast Asia, Indonesia, Sri Lanka, northern Australia, as well as Madagascar, the Seychelles, the Maldives and Mauritius [4]. Breadfruit is regarded as an energy food, a source of complex carbohydrates, vitamins and minerals [5]. Breadfruit bears fruit with edible dry mass up to 6 t/ha, comparing favourably with other common staple crops, and has been recognized for its potential to alleviate hunger in the tropics [6]. Breadfruit makes a significant contribution to the local food security, often a major tree crop within an indigenous agroforestry system which can be grown sustainably with relatively low agricultural inputs [3].

Despite its importance as a traditional food security crop and its emergence as a commercial crop [7], breadfruit cultivation encounters several constraints in the tropical regions. These include susceptibility to natural disasters, such as cyclones and hurricanes and prolonged drought [8]. Breadfruit is also susceptible to some pests and diseases, namely the fungi, *Phytophthora palmivora* and *Phellinus noxius* which cause rots. *P. palmivora* affects the fruit and *P. noxius* the trunk and root, eventually killing the tree [9]. *Bactrocera frauenfeldi* can also attack breadfruit and in combination with *B. umbrosa* affected 75% of fruits in Papua New Guinea [10]. Climate change is likely to exacerbate the impact from extreme weather and natural disasters according to current projections [11]. However, it is not clear how global climate change will affect these pests and diseases in breadfruit cultivation.

Being an evergreen tree from 15-30m, breadfruit is prone to wind damage [1,12]. During the past decades, many atolls and high islands have experienced destructive cyclones which can have a devastating impact on islands that rely heavily on breadfruit for a staple food. For example, in 1990, Cyclone 'Ofa' destroyed 100% of the breadfruit crop in Samoa, and 50 - 90% of the big mature trees were blown over [13]. Almost entire breadfruit crops were lost in Upolu after Cyclone 'Evan' hit Samoa in 2012 [14]. Cyclone-related tree loss was also responsible for a reduced number of fruiting trees in Fiji [9]. Similarly in the Caribbean, a major breadfruit producing region, hurricanes in the 1990s resulted in the loss of and damage to a significant number of breadfruit trees [13]. In the 1980s an estimated 50% of the breadfruit trees in Jamaica were killed or damaged by windstorms [15]. The continuing trend of global climate change with more intense hurricane-force storms will have serious implications for island nations throughout the Pacific and Caribbean [8,11].

Tree height is also a major constraint to disease control and fruit harvesting. Fruit production costs increase as trees grow taller. This is due to the high labor cost for ongoing pruning, removal of dead or diseased branches, and challenging harvest. Mechanical harvesters

have not been utilized for most of breadfruit cultivation, therefore labour intensive (frequent climbing) remains the only viable option [3]. This method of harvesting results in a high proportion of either damaged fruit, or fruit with a limited shelf-life after falling to the ground during harvest. It is estimated that over 50% of the fruit may be lost due to the difficulty of harvesting from large trees [16].

Pruning is an option often used to reduce tree size, however it has its drawbacks. Severe branch pruning can reduce yield because the trees are stimulated to grow more vigorously in the subsequent season. Poor pruning practices may have severe effects on the health of a tree, this can lead to wound injury and invasion of fungal pathogens [9]. There is little investment in breadfruit. Being an understudied crop, information on the agronomy, pruning and orchard management of breadfruit is currently limited [9].

In response to these constraints there is increasing interest in developing breadfruit varieties with short-stature [17]. The benefits of dwarfism in fruit-tree industry have been clearly demonstrated with the widespread use of dwarfing rootstocks in apple and peach [18,19]. Today, breeding efforts have resulted in selection of dwarf scions or dwarfing rootstock varieties in almost all of the main temperate and tropical fruit species [20]. These commercially acceptable dwarf varieties have revolutionised fruit production by allowing dense field cultivation, increasing harvest index and substantially decreasing production costs [21,22]. Building on a going body of research contributing to our understanding of plant dwarfism, the present review discusses the opportunities and challenges toward breadfruit dwarfing, with focus on issues related to strategies and prospects of natural and induced genetic variability, horticultural techniques, molecular breeding and the potential role of genomic tools for the development of dwarf phenotype.

2. MECHANISM OF DWARFISM

2.1 Gibberellin

Various factors cause dwarfism in plant, of which gibberellin (GA) and brassinosteroids (BRs) are the most important factors in determining plant height [23,24]. Research from rice, barley and *Arabidopsis* mutants has demonstrated that dwarfism is commonly associated with deficiencies in GA levels or signalling [25,26]. The level of bioactive GAs in plant is controlled by several mechanisms, including transcriptional regulation of genes encoding enzymes for GA biosynthetic and catabolic pathways. The GA biosynthetic genes were negatively regulated by high GA levels whereas the GA catabolism genes were positively regulated by the GA concentrations [27,28]. GA promotes plant growth by inducing the degradation of DELLA proteins which act as GA signal repressors [25,29]. DELLA proteins consist of N-terminal amino acids, D-E-L-L-A (DELLA domain) essential for perception of the GA signal and a C-terminal region for repressor function [25,29]. Shortly after gibberellin stimulation, DELLA proteins are degraded in the plant. However, mutant DELLA proteins are resistant to destruction and accumulate to cause dwarfing phenotype by constitutive growth repression [30]. Therefore by modifying regulation of genes controlling GA flux and GA response, it is possible to modify processes regulated by GA and, thus, plant form [31]. These apply to dwarf or semi-dwarf varieties of the wheat reduced height-2 (*rht*), the rice semi-dwarf-1 (*sd1*), the maize dwarf-8 (*d8*), the rice *gai* and the barley slender1 (*sln1*) [26].

Recently, it has found that jasmonic acid (JA) can antagonize the GA mediated response through modulating the levels of DELLA proteins [32]. *Arabidopsis* mutants over-producing

JA have stunted stems, and a rice semi-dwarf mutant *rim1-1*, displaying resistance to rice dwarf virus, has high expression level of genes encoding JA biosynthetic enzymes, leading to a rapid accumulation of JA after wounding [33]. In this sense, interaction between GA and JA signalling is used to make a balance between “growth” and “defence” in response to various stimuli [34].

2.2 Brassinosteroids

Brassinosteroids (BRs) are a class of plant steroid hormones that promote plant growth and regulate organ morphology through controlling cell elongation and division. They are also important for vascular differentiation, flowering, light responses, and regulation of other hormone signalling, particularly the auxin pathway [35]. BRs are produced from campesterol by a network of reactions, the genes responsible for each reaction are not completely known [36]. BR-related mutants usually exhibit short and compact stature with deep green and erect leaves and delayed flowering [37,38]. The rice *Osdwarf4-1* mutant exhibits erect leaves and slight dwarfism without compromising grain yield [39]; this phenotype is due to loss of function of a cytochrome P450 involved in BR biosynthesis [40]. A dwarf brassinosteroid-deficient mutant of broad bean (*Vicia faba* L.) created by γ -ray irradiation was defective in sterol C-24 reduction (the metabolism of the sterol 24-methylenecholesterol to campesterol) [41]. The barley semi-dwarf mutant carries a mutant allele of a gene encoding a putative BR receptor [37]. Manipulating BR biosynthesis and signal transduction is a strategy for generating dwarf phenotypes [38].

3. GENETIC RESOURCES FOR DWARFING CLONES

3.1 Reproductive System

The genetic structure of a plant species is largely influenced by its reproductive system. Breadfruit is considered as an out-crossing species [1], but the species is monoecious, with self pollination prevented by a temporal separation due to the male inflorescences appearing earlier than female inflorescences [42]. Breadfruit comprises fertile and sterile diploids ($2n = 2x = 56$) and sterile triploids ($2n = 3x = 84$) [1,43]. Significant morphological variability exists including true seedless varieties, varieties with several aborted seeds, and those with numerous viable seeds [12]. Fruit development in seedless breadfruit is parthenocarpic and does not require pollen to be initiated [44] and in fact, little is known about pollination in seeded cultivars with both wind and insect pollination being suggested [3].

The seeded, out-crossing, fertile varieties are mostly found in the western South Pacific, while the seedless forms predominate in the eastern islands of Polynesia [43,45]. Molecular evidences based on amplified fragment length polymorphism (AFLP) have suggested that the Melanesian and Polynesian cultivars, *A. altilis*, may have been derived from *A. camansi*, whereas the Micronesian cultivars may be the product of interspecific hybridization between the *A. camansi* –derived cultivars and *A. mariannensis* and subsequent introgression [44,45]. Frequent recombination and segregation events have contributed to the genetic diversity of the domesticated breadfruit during thousands of years of evolution [44]. At the same time, repeated vegetative propagation has played a role in fixing heterozygosity and maintaining the unique gene combinations that confer the specific phenotypes. This is evident by the high degree of morphological diversity and many distinct cultivars specific to particular island groups [44].

Vegetative propagation is required for seedless varieties and preferred for seeded varieties [3]. Seeds are rarely used as true-to-type seedlings rarely occur, and it is difficult for viable seeds to survive desiccation [1]. Clonal propagation is generally through root suckers, root cuttings, or air layering [1,12]. Seedless varieties can be grafted onto seeded rootstock using various techniques such as approach grafting or cleft grafting [3].

3.2 Breeding

Deliberate breeding of breadfruit has not been reported. Indigenous islanders have selected seedlings or somatic variants from natural populations for desirable characters over thousands of years [1], but selection has not been rigorous in most areas where breadfruit is cultivated. Many of the Pacific Island cultivars have been present for generations. Generally few new cultivars are recognized and selected, particularly where seedless and few-seeded cultivars predominate – in these locations islanders typically rely on a group of preferred cultivars, because they are well-adapted to that location and grow and fruit well [1]. Seedling trees are retained on occasion but rarely multiplied. In limited few areas, such as Santa Cruz Islands, where breadfruit forms important part of traditional arboriculture systems and most cultivars have seeds, seedlings are allowed to grow until they bear fruit. New seedlings with desirable traits are selected and maintained by vegetative propagation [1,46].

3.3 Genetic Diversity and Dwarfing Rootstocks

Through horticultural practice, scions may be dwarfed by grafting onto dwarfing rootstocks. The dwarfing effect of the rootstock may also be induced by a stem piece or interstock (intermediate stock) grafted between a scion and rootstock [19]. Apart from greatly reducing the vigour of the grafted scion, the practice has revolutionized the production of some perennial tree crops by shortening the time to flowering (juvenile phase) [22]. Apple seedlings have a long juvenile period and can take 4–8 years to flower and grafting the scion onto dwarfing rootstocks shortens this period by several years [47]. Similarly, grafted breadfruit trees can begin bearing in 2-3 years, while vegetatively propagated trees start fruiting in 3-6 years, and trees grown from seed may begin to produce fruit in 6-10 years [9].

The wealth of genetic diversity of breadfruit that exists is the source for future development of dwarf rootstocks or scions. 132 cultivars from Vanuatu, 70 from Fiji, 50 from Pohnpei, more than 30 from Tahiti and over 40 from Samoa have been documented [3]. The genetic variability has resulted in enormous phenotypic variation in morphological, agronomic, and nutritional characteristics among cultivated varieties [44]. AFLP data has been used to understand the relationship between breadfruit *A. altilis* and its wild relatives, *A. camansi*, and *A. mariannensis* [46]. Recently, phylogenetic classification based on chloroplast and nuclear DNA sequences has provided insight into the diversity of inflorescences and infructescences of the genus *Artocarpus* and the family Moraceae in an evolutionary context [48]. Research has also been carried out to develop high polymorphism molecular markers, such as microsatellite loci to facilitate phylogenetic analyses, cultivar identification and germplasm conservation of breadfruit [49].

A dwarf variety of breadfruit was reported at the Pacific island of Niutao [50]. However, the agronomical characteristics of the variety are largely unknown. In addition, two other cultivars, 'Ma'afala' and 'Puou', popular in Samoa and Tonga, tend to be shorter and more compact than most other varieties. They are commonly seen as small trees up to 10 m with dense spreading canopy at their local regions [51]. Detail information on tree architecture of

many traditional Pacific Island cultivars is currently limited and it may be possible that naturally dwarf cultivars with desirable agronomical traits can be identified through in-depth characterisation and vigour selection.

Dwarfing rootstocks may also come from species related to breadfruit. Grafting breadfruit on *A. camansi* rootstock has been reported [52]. Though species of *Artocarpus* genus are mostly tall tree and rarely shrubs, they display great diversity in the tree stature. For example, *A. anisophyllus* and *A. hirsutus* are large rainforest trees up to 50 m [53,54], species like *A. camansi* (breadnut), *A. nitidus*, *A. mariannensis* (dugdug), *A. integer* (chempedak) and *A. heterophyllus* (jackfruit) tend to be a medium size tree to 25m, with other species, such as *A. lakoocha* about 10 ~ 15m, and *A. petelotii* up to 10 m [46,55-57]. Noticeably, species *A. xanthocarpus* is reported to be up to 8m [55].

The vigor of shoot growth and eventual size of tree at maturity is usually controlled by the use of rootstocks while the scions also have a significant effect on final tree size. Grafted *A. heterophyllus* (jackfruit) trees were found to have a dwarfing tendency [58], with cultivar "Ziman Pink" marketed as a dwarf type of jackfruit [59]. The effect of grafting on tree vigor, particularly the choice of rootstocks on tree stature of breadfruit cultivars is worthy of experimental investigation.

4. DWARFING BY CHEMICAL TREATMENT

Plant growth retardants are widely used in agricultural industry. Many species, including cereals, grasses, fruit trees and ornamentals, are regularly treated with chemicals to control plant stature [60]. Most of these growth regulators act as GA biosynthesis inhibitors. To date, four different types of GA inhibitors are known: 1) Onium compounds including chlormequat chloride, chlorphonium and AMO-1618 (2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidinecarboxylate methyl chloride); 2) N-containing heterocyclic compounds including hexaconazole (HX), ancymidol, flurprimidol, tetcy-clasis and paclobutrazol; 3) Acylcyclohexanediones including prohexadione-calcium (Pro-Ca), trinexapac-ethyl (TNE) and daminozide; 4) 16, 17-dihydro-GA5 and related structures [61]. Some chemicals such as daminozide, ethephon and paclobutrazol are persistent in the plant as an un-metabolized form and therefore have raised concern due to the residue toxicity and health risk [62]. Recently, chemicals like Pro-Ca, TNE and HX represent a novel class of plant growth regulators that show a lack of persistence in plant. The short-term effect of these chemicals provides a flexible tool for vegetative growth management that can be applied at different times and growth strategies [63].

Pro-Ca has gained attention not only for its specific inhibitory effect on seedling height and shoot length without any residual problems in the plant and soil, but also for increasing yield, fruit quality and fruit set of some species such as tomato, strawberry, pear and avocado [64]. Pro-Ca is a structural mimic of 2-oxoglutaric acid, a co-substrate of dioxygenases that catalyze late steps of GA biosynthesis, therefore blocking 3 β -hydroxylation and inhibiting the formation of active GAs which leads to the suppression of shoot elongation and a more compact canopy [61]. The chemical was shown to have a similar effect to another GA biosynthesis inhibitor, chlormequat chloride (CCC), but with low toxicity and limited persistence [65].

Pro-Ca has been registered in the U.S.A and Europe for use on apple [65], rice [63], petunia and okra plants [66], Camarosa strawberry [67], sorghum [68] and peanut [69]. Application

of Pro-Ca reduces the length of stem internode and vegetative growth of fruit trees including apple [70], pear [62] and cherry [71]. Generally, the rate needed for effective vegetative control has to be raised as the vegetative vigour of the trees increases [63]. The compound has been reported to have no negative effect on yield, fruit quality, fruit set and flower initiation [62,70,72], although delayed initiation of flowering or fruit set has been reported in several studies [66]. Pro-Ca significantly shortened the annual shoots of a walnut cultivar [73], and greatly suppressed the stem length of peanut [69] and sweet sorghum crops [74]. Pro-Ca does not persist in the plant therefore does not directly affect vegetative growth in the following season [63]. In higher plants, the chemical degrades to a natural product through deacylation and ring cleavage with a half life of a few weeks, whereas in the soil, Pro-Ca decomposes mostly to carbon dioxide, with a half life of <7 d [63]. No negative effects have been reported on birds, fish, honey bees (*Apis mellifera* L.) or soil micro-organisms [63].

The use of plant growth regulators in breadfruit trees has not been reported. It needs to investigate the response and sensitivity of these chemicals to the tree stature of breadfruit cultivars and their persistence. Given that GA affects many developmental processes including shoot elongation, flowering and fruit set [27,75], fruit size and even postharvest fruit quality [76-78], the use of GA inhibitor has showed different response depending on the timing, cultivars and rate used [79]. Further commercial use of these chemicals in breadfruit trees should also consider the tropical climate, fertilization and the multi-cropping system used in the cultivation. There would also be concern that the use of these chemicals would conflict with the general move in the Pacific for produce to be organic or as chemical free as possible.

5. DWARFING BY INDUCED MUTATION

Genetic resources showing dwarfing characteristics are rare in some fruit trees, including breadfruit, and breeding dwarf rootstocks in such species is difficult. Genetic improvement of breadfruit cultivars by conventional breeding can be a very slow process due to their long juvenile phase and heterogeneous genetic background. Alternatively, genetic variability can be induced by mutagenizing agents, such as chemical and physical mutagens [80-82]. Induced mutations are random changes in the nuclear DNA or cytoplasmic organ, resulting in chromosomal or genomic mutations that enable plant breeders to select desirable traits [83]. The technique has potential for modifying existing traits or creating new valuable traits within the cultivated varieties [84]. It is widely used for crop improvement in both seed and vegetatively propagated crops, with currently more than 3200 officially released mutant varieties from 214 different plant species throughout the world [85]. Mutation breeding is being investigated in tropical fruit trees, such as litchi, guava, cherimoya, pitanga, jaboticaba and carambola [86]. These species are all characterised by long juvenile phases which make conventional breeding programmes slow and costly [86]. In this context, the techniques can be used in breadfruit to overcome the long growth cycle and triploidy problems and produce materials that can be screened for advantageous traits such as dwarf phenotype. The putative dwarf mutants identified by mutagenesis can be further used in breeding programs as parental germplasm materials or rootstocks for grafting.

5.1 *In vitro* Mutagenesis

The combination of *in vitro* culture with mutagenesis (*in vitro* mutagenesis) offers an efficient method for handling large populations, therefore increasing the efficiency of mutation

induction, mutant recovery and selection [84]. *In vitro* mutagenesis in breadfruit is now feasible due to a robust *in vitro* culture system established for the species [87-90]. At least 17 cultivars of breadfruit have been successfully *in-vitro* propagated [3]. The protocol involves shoot proliferation, rooting of regenerated shoots and the establishment of rooted plantlets in the greenhouse [88,89]. During the procedure, small pieces of breadfruit plant tissues, such as auxiliary shoot tips and small buds are surface sterilized and cultured on a growth medium under aseptic conditions. Optimization of the type and concentration of plant growth regulators in the culture medium induces the proliferation of shoots or roots. After root differentiation, whole plantlets are potted and acclimatized in growth chamber before transferring to greenhouse [88,89]. *In vitro* culture has the advantage of rapid multiplication and distribution of true-to-type planting material on a large scale and all year round [90,91].

The procedure of *in vitro* mutagenesis is rather straightforward: isolated explant tissues are either soaked in a chemical mutagen for a prescribed amount of time, or exposed to a pre-determined dosage of ionizing radiation. After mutagen treatments, tissues can be subjected to standard regeneration procedures [92]. It is important to note that in comparison with approaches associated with single cell origin procedures, such as somatic embryogenesis, mutagenesis on multicellular tissues including meristem tissues, offshoots and seeds leads to chimaeras composed of non-mutated cells and cells with different mutations. Repeated rounds of vegetative propagation must be carried out to dissociate chimeras [83,93,94]. The mutagenesized tissues (M1V1 generation) are typically required to undergo at least three culture cycles (M1V3) to eliminate chimeras [95,96].

In vitro mutagenesis was used to induce mutation for selection of reduced-stature and flower colour in *Rosa spp.* [97-99], streptomycin-resistance in *Solanum surattense* [100], salt tolerance in *Chrysanthemum morifolium* [101] and various morphological characters in *Lilium longiflorum* [102]. The techniques have successfully been used to improve agronomic characters such as tree stature, fruit yield and disease resistance of a variety of fruit crops (Table 1), including selection of 98 dwarf clones from 10,000 mutant banana plants [103].

Table 1. Examples of *in vitro* mutagenesis for improvement of fruit trees

Species	Mutagens	Selected traits	Explants	References
<i>Pyrus communis</i>	gamma rays	compact trees, fruit traits	<i>in vitro</i> shoots	[104,105]
<i>Prunus salicina</i>	gamma rays	vegetative and fruit traits	<i>in vitro</i> shoots	[96]
<i>Phoenix dactylifera</i>	gamma rays	disease resistance	somatic, embryogenic cells	[83]
<i>Citrus</i>	gamma rays	seedless fruit	shoots	[106,107]
<i>Psidium guajava</i>	gamma rays	disease resistance, fruit characteristics	shoot tips	[86,108]
<i>Ziziphus jujuba</i>	gamma rays		<i>in vitro</i> shoots	[109] [96]
<i>Musa spp.</i>	EMS	disease-resistance, reduced height	shoot tips, axillary buds, microsections	[95,103,110]
<i>Beta vulgaris</i>	gamma rays	drought-tolerance	<i>in vitro</i> shoot tips	[111]
Strawberry	gamma rays	morphological characters	<i>in vitro</i> buds	[112]
<i>Solanum lycopersicum</i>	EMS	various traits	<i>in vitro</i> cuttings	[113]

Different mutagens can produce different spectrums of mutations [114]. The most commonly reported chemical mutagen, ethyl methanesulphonate (EMS), causes primarily GC-AT base pair transition mutations in many species, leading to single nucleotide changes [115]. While the majority of induced point mutations are predicted to be functionally silent, other nucleotide changes such as non-sense, mis-sense, RNA splicing defects and regulatory alterations can have varying effects on gene expression and protein function [116]. On the

other hand, physical mutagens have shown potential for application in fruit breeding. Among these, ionizing radiations (X-rays and gamma-rays) have been widely used in fruit trees [84]. Ionizing radiation can induce a series of mutations ranging from point mutation to mitotic recombination, or gene conversion and chromosome aberration, therefore generate many kinds of phenotypes including homozygosity [117,118]. Although point mutation rate with gamma rays can be lower than that with EMS, the rate of knockout mutations is usually higher [119].

5.2 Mutation Induction on *in vivo* Plant Materials

Mutagenesis can be carried out on seeds and vegetative materials. Due to the chimeric nature of mutation on multi-cellular tissues, the phenotypic changes in the treated generation (M1) are potentially not heritable as only a subset of cells are involved in gametogenesis [120]. This issue can be overcome by performing at least one sexual cross prior to analysis. Mutagenesis on seeds is therefore relatively straightforward for seed-propagated plants that are capable of self-fertilization, as any chimerism is eliminated through sexual reproduction [93]. In this manner, seeds treated with different dose of EMS are planted (M1 generation), and a total of ~ 2000 M1 plants are self-fertilized to grow M2 generation. Most mutant alleles are recessive, so successful mutants would not be seen until the M2 generation [121], however, it might take a long time to grow out M1 generation. Lethal mutation and infertility will often significantly affect the population. Using gamma ray irradiation on seeds, Badigannavar and Suvendu (2009) identified dwarf mutants of groundnut (*Arachis hypogaea* L.) where the plant height was reduced by 24.5% to 41.0%. Progenies from a selected dwarf clone were consistently segregated into dwarf, extreme dwarf and parental types [122].

While seed mutagenesis can be applied to some fertile diploid breadfruit cultivars, the long growth cycle can make the process slow and costly. The out-crossing nature of breadfruit can also compound the difficulty of mutant identification, and the self-fertilizations necessary to identify mutants in the population can result in reduced plant vigour as a result of the genetic background and not necessarily the mutations.

Mutagenesis directly performed on shoot cuttings applies to both seed and vegetatively propagated crops. Irradiation is more widely used as a mutagen [84], after irradiation treatment, plant materials may be sub-cultured by cutting or used as scions and immediately grafted on other mature rootstocks [86]. This method was used for improving citrus [106,107] and guava [108]. Similar experiments have also been carried out on litchi, carambola, cherimoya, pitanga and jaboticaba [86].

5.3 Mutant Detection

5.3.1 Phenotyping

The identification of desired mutants should be performed on nonchimaeric plants. An affordable, high throughput phenotypic screening method for the traits of interest must be in place to process the large number of individual lines. These include a fast analysis of plant height, internode length and number, shoot and lateral shoot length and diameter for detection of dwarf phenotypes in a population of several thousands or tens of thousands of mutant plants [105].

Owing to lack of meiosis to assort mutation during vegetative propagation, induced mutations are heterozygous, meaning only dominant or hemizygous alleles will likely yield phenotypes of interest [114]. This is particularly challenging if the mutated cultivars are polypoids. In these species, most genes are represented by multiple homoeologous copies, and phenotypic effects in a single mutant may be masked by wild-type homoeologues present in another genome. This not only raises doubts as to the effectiveness of the facile screen of plant height for the outcome of mutagenesis, but also implies that in some cases, combined mutations by developing double or triple mutants through genetic crosses is required [123].

5.3.2 High throughput genotyping

Technologies allowing rapid and automated identification of mutations in targeted sequences are emerging. One of these techniques is TILLING (targeting induced local lesions in genomes) [124,125]. In this method, genomic DNA samples from a population are pooled, arrayed on microtiter plates and subjected to gene-specific PCR with fluorescent-labelled primers. The amplification products are incubated with an endonuclease that recognises and cut mismatches in heteroduplexes. The cleavage products are electrophoresed and visualized by fluorescence detection. The migration of cleaved products indicates the approximate location of nucleotide polymorphisms. Upon detection of a mutation in a pool, the individual DNA samples are similarly screened to identify the plant carrying the mutation [124,126]. TILLING technology has been developed for efficient identification of induced point mutations that are generated by chemical mutagens, such as EMS in a large population, and has also shown great potential for screening population treated with gamma ray and X-ray [119,127]. A variation of the TILLING method, known as EcoTILLING, has also been developed for detection of natural (allelic) variation in the germplasm [126]. Techniques that do not rely on mismatch cleavage by endonucleases have also been developed. This alternative method employs the high-resolution melt analysis (HRM) to identify single-base mismatches in the mutant pools by analysing a shift in the melting temperature of the heteroduplex in the PCR products [128]. Recently, TILLING by sequencing, the application of next-generation sequencing (NGS) in TILLING, has provided a platform to screen mutant populations or germplasm collections in a high-throughput fashion. Rigola et al [129] first reported the KeyPoint technology which employed amplicon sequencing with multidimensional sample pooling and barcoding, and identified two mutants in a 287 bp targeted region from 15,000 plants representing 3008 families of M2 tomato plants [129]. Obviously, the success of the technology in identifying dwarf mutants will depend on the right selection of target genes. Numerous studies on plant dwarfism have led to the discovery of a few dwarfing candidate genes for crop improvement. These include orthologs of the wheat with reduced height-1 *rht* gene and the rice semi-dwarf *sd1* gene, both encoding DELLA proteins, an important components of GA signalling, and those of *sd1* encoding key enzymes in the GA biosynthesis pathway [25,29]. Using this high throughput platform, Zhu et al. recently performed screen at these dwarfing candidate genes to identify semi-dwarf mutants in Tef (*Eragrostis tef*), an under-studied species [123]. Following sequenced with Roche 454 sequencing technology, they identified several mutant lines with mutation in the target regions from an EMS-mutagenized population consisting of 21,210 individuals [123].

The dwarf traits of the breadfruit species and its genetic basis have not been characterized. As there is limited genome sequence for the species, sequences of all homoeologues related to dwarfing candidate genes have to be obtained and annotated before the TILLING screen can begin. Strategies used to obtain these target sequences include screening and

sequencing cDNA or genomic library, and gene cloning by degenerate PCR. Once sequences information and genomic structures of the dwarfing candidate genes are available, efficient gene specific primers can be designed for TILLING screening of indels or inter-homoeologue SNPs for target loci [130].

6. DWARFING BY TRANSFORMATION

Plant transformation involves introducing and stably expressing a segment of DNA into a plant [131]. The process includes integration of DNA sequences into the genome of cells capable of giving rise to a whole plant [131,132]. Owing to the fact that natural selection will act to remove dominant alleles that result in dwarf stature in the face of competition for light, healthy dwarf genotypes are expected to be rare, and difficult to obtain through traditional tree breeding [20]. Therefore, insertion of dominant transgenes may be an alternative method for obtaining dwarf phenotypes in many species [60]. Increasing or decreasing GA levels has been explored in several species, by over-expressing or silencing genes involved in GA biosynthesis pathway [60]. Over-expression of GA2ox, a GA catabolism enzyme caused dwarfism in tobacco and poplar [133,134]. In poplar, over-expressing a gene encoding DELLA protein, a repressor of GA signalling caused severe dwarfism and increased root proliferation [134,135]. Transgenic kiwifruit plants over-expressing a isopentenyl transferase gene to control endogenous cytokinin levels were also used as a rootstock directly grafted onto wild-type seedlings to produce dwarf phenotypes in the grafted plants [136].

There are various methods available for gene transferring, such as *Agrobacterium*-mediated transformation, particle bombardment, electroporation and viral transformation [131]. In all case, single cells are transformed and regenerated into whole plants by *in vitro* culture procedures [131]. The first requirement for transformation is an efficient regeneration protocol. As previously discussed organogenesis has been developed for many breadfruit cultivars providing efficient regeneration from auxiliary shoot tips and small buds [89]. There is currently no report on somatic embryogenesis of breadfruit. Somatic embryogenesis is usually preferred for transformation owing to its possible single cell origin, and less chance of getting chimeric plants [131]. However, multiple rounds of shoot regenerations as demonstrated by previous experiments [131,137] provide feasible strategy to achieve a significant reduction of chimeras and purify isogenic transgenic lines.

While the technique for *in vitro* culture of breadfruit is available, an efficient gene transfer protocol through *Agrobacterium*-mediated transformation or particle bombardment needs to be established. This process can create a substantial barrier to the application of transformation in some crops [132]. Compared to annual crops, woody trees such as fruit trees were considered recalcitrant to genetic transformation and regeneration for a long time, however through persistent efforts, they now begin to show enormous progress in many species including apple, oranges and grapefruit [138,139].

Recently, "intragenic or cisgenic plant" represents a concept, wherein the gene, promoter and terminator sequences derives from the same crop or from sexually compatible species to generate designer crops with more public acceptability [140]. For this to happen, the indigenous genes of the target species and their regulatory sequences need to be characterized [141]. There is hope that the expanding of genome sequencing and proper annotation to many non-model plant species would help in gene isolation and utilization for the development of these new generation of transgenic crops.

7. CONCLUSION AND FUTURE PROSPECTS

Agricultural solutions are required to reduce breadfruit tree stature in order to withstand cyclones/hurricanes and improve harvesting efficiency. The wealth of genetic diversity among breadfruit cultivars provides an important reservoir of genetic variation for developing dwarfing rootstocks. Phenotypic studies facilitated by Next Generation Sequencing (NGS) are generating a large volume of genomic and transcriptomic information for use as functional markers or breeding targets in many species with limited genome resources [142]. Morphological diversity in tree height and canopy shape exists in breadfruit cultivars throughout Oceania, but quantitative analysis is required with a focus on the tree architecture, yield and the resistance to wind damage. Genome-wide surveys are needed to improve our understanding of the genetic capacity of the diversity that exists, and to develop sequence-based genetic markers to assist in breeding programmes and early detection of dwarf traits [143].

When suitable dwarf lines are not available, chemical treatment is commonly used as an alternative to the high labor cost of mechanical pruning. Chemical Pro-Ca has shown considerable promise for use on some fruit trees due to its low persistence in plant and soil. Its responses and sensitivity however have not been tested on breadfruit cultivars. While it may have potential for reduction in tree height in the short term, application of such chemicals to vigorous fruit trees every growth season can be costly, and may not be sustainable in the long term. The development of operations that incorporate the relatively intensive use of chemicals would risk reducing opportunities for Pacific farmers to capture niche markets based on chemical-free/organic production.

In vitro mutagenesis provides an attractive solution for the development of dwarf varieties. The method would preferably be applied in diploid cultivars since loss-of-function mutations in triploids are less likely to produce a phenotype due to gene redundancy and lack of meiosis in successive rounds of tissue culture. Mutation in dwarfing candidate genes can be screened by PCR markers for fast detection. The application of NGS and TILLING has provided high-throughput screening tools for mutation discovery without the need for an expressed phenotype.

Plant transformation has shown potential of introducing dominant dwarf traits into plant species through manipulating GA-related genes. However, even though the transformation techniques would work on breadfruit species, the method has a long way to go before gaining acceptance from both local consumers and the export market.

There is lack of information about the breadfruit genome. The species has a relative small genome estimated as ~ 880 Mbp/C for diploid cultivars, Puou and Maafala [88]. It is an attractive candidate for genome analysis initiatives and development of genomic tools for breeding programmes. With large number of genomes being sequenced or resequenced in the last several years [144], there is hope that as new technologies are developed, assembly of *de novo* genome sequences in many less-characterized species like breadfruit will become possible. The availability of the complete genome sequence will rapidly increase molecular marker resources and their application to breeding and selection for important traits, such as dwarf characters, stress and pathogen resistance in breadfruit cultivars.

The recent IPCC report (2013) confirmed that climate change will have a negative impact on global food staples [145]. For those countries in the Caribbean and Pacific regions which are largely dependent on imported foods for their food security – these projections are delivering

a warning as to the likelihood of future increased costs and instability in supply. The time is right therefore to invest in staple food crops such as breadfruit to substitute for imported staples. Strengthening breadfruit production so that continuity of supply is linked to processing developments is very important for the future food security of these island regions. Export opportunities also exist, reinforcing the need for increased investment and research into areas like dwarfism that would facilitate development of commercial systems. This review has described several approaches that could be used to generate dwarf breadfruit trees. Understanding and analysing the wealth of diversity that exists in the Pacific is clearly the first step in the process – farmers, extensionist and scientists all have a role to play in this process. However, successfully developing an efficient and effective tool for breadfruit dwarfing can only be achieved through partnerships with institutes capable of delivering that technology.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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