Mass-Propagation and Bioreactor-Based Technologies for Germplasm Conservation, Evaluation and International Distribution of Breadfruit

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Abstract

It has become increasingly important to facilitate re-distribution and access to breadfruit cultivars that have been selected over millennia by the indigenous peoples of the Pacific region but may have been lost from many local communities. The collection of breadfruit cultivars at the Breadfruit Institute of the National Tropical Botanical Garden (NTBG) provides an important germplasm resource, but redistribution from the site has been hampered by: 1) the relatively few number of trees for each individual cultivar; 2) the limited number of roots available to provide root cuttings or root shoots from each tree; 3) international plant quarantine requirements that limit the distribution of root cuttings; and 4) the low success rate for establishing trees from mature roots. Over the last three years, an efficient micropropagation and plant production strategy was developed for in vitro clonal propagation, regeneration, large-scale bioreactor production and acclimatization of mass-propagated breadfruit plantlets into tropical environments.

INTRODUCTION

Mass-propagation and regeneration of any species relies on the fundamental principles of regulating plant growth and development (Skoog and Miller, 1957). In this basic hypothesis, the redirection of plant growth is primarily dependent on the relative concentrations of two classes of phytohormones, viz. auxin and cytokinin with other growth substances essential for the process in some species. Over the last 50 years, this hypothesis was validated with innumerable experimental systems and formed the basis for modern plant breeding and genetics programs (Murch and Saxena, 2005).

Micropropagation is the process by which each individual plant cell can be induced to form a whole plant, a phenomenon known as totipotency (Krikorian, 2005). Micropropagation protocols proceed through a series of stages first described by Murashige (1974) and later modified by Debergh and Maene (1981). The objective of micropropagation protocols is to produce disease-free, true-to-type plants beginning with the selection of stock plants representative of the genotype, healthy and vigorous (Stage 0), followed by the establishment of meristem cultures which are normally virus-free and relatively easy to establish in vitro (Stage 1), in vitro multiplication of shoots resulting in masses of clones of the original plant (Stage 2), rooting of the in vitro regenerated shoots (Stage 3), and finally, ex vitro acclimatization of whole plants (Stage 4); reviewed in Cassells (2005).

Despite its economic significance, only a few studies have been published describing micropropagation of breadfruit. In vitro cultures of a yellow cultivar have been

established in the Caribbean (Rouse-Miller and Duncan, 2000) and micropropagation of three cultivars in the collection at National Tropical Botanical Garden (NTBG) was recently reported (Murch et al., 2007). These studies describe the establishment of relatively few cultivars in vitro and many other cultivars that are recalcitrant in the same regeneration protocols. Once in vitro cultures are established, methods are needed for increasing the speed of regeneration, efficiency of root initiation and acclimatization to ex vitro environments, and must be optimized for each cultivar. This paper describes the in vitro establishment of two additional cultivars that were previously recalcitrant and the methods for rapid, efficient rooting and bioreactor culture systems for large-scale breadfruit production.

MATERIALS AND METHODS

Stage 0: Selection of Stock Material

Germplasm accessions 'Ulu fiti' (Accession 900260001) and 'Pua'a' (Accession 890460001) from the NTBG breadfruit field genebanks located at Kahanu Garden in Hana, Maui and at the McBryde Garden in Lawai, Kauai, United States, were used for this study because of their desirable agricultural characteristics. Root cuttings were taken from mature trees in November 2006, thoroughly washed and drenched with fungicide before shipment to the University of British Columbia Okanagan laboratories. Root cuttings were established in a sterilized soil media in a growth chamber at 25°C with a 16 h photoperiod and watered weekly until budbreak.

Stage 1: Establishment of First Generation Cultures via Meristems

Based on previous studies indicating mature buds were not suitable for establishing in vitro cultures of these cultivars, the first buds to break on the root cuttings as they were released from dormancy were excised and surface sterilized with 70% ethanol for 1 m and 10% bleach (5.25% sodium hypochlorite) for about 15 m before washing several times with sterile distilled water. The sterilized tips were cultured in tubes containing 22 ml of culture medium. The *Artocarpus* shooting (AS) medium is comprised of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 2 µM benzylaminopurine (BA), 3 µM kinetin, 3% sucrose with pH adjusted to 5.75 prior to the addition of 0.25% gellam gum (Sigma Chemical Co., St. Louis, Missouri, USA) and autoclaving. The combination of phytohormones was selected from initial experiments comparing a range and combinations of BA and kinetin concentrations (1-10 µM) (Murch et al., 2007). Cultures were maintained at 28°C in 16 h photoperiod (25-40 µmol m^{-2} s^{-1}) provided by cool white fluorescent tubes.

Stage 2: Shoot Multiplication

After 3-4 w of culture on AS medium, developing shoots, about 0.5 cm long, were transferred into Magenta boxes, each containing two shoots, following protocols established for breadfruit cultivars 'Ma'afala', 'Puou' and 'Puupuu' (Murch et al., 2007). Cultivars 'Ulu fiti' and 'Pua'a' failed to develop further on the standard media. Therefore, multiple experiments were initiated comparing the efficacy of various growth regulators, including indoleacetic acid, indolebutyric acid, 2,4-dichloroacetic acid, thidiazuron and gibbrellic acid at a range of concentrations (0-15 µM).

Stage 3: De Novo Root Initiation

Regenerated shoot explants (2 cm in length with two nodes) were excised from the regenerating plantlets and subcultured onto a medium containing MS salts, B5 vitamins, 3% sucrose, indoleacetic acid (IAA; 0-10 µM) or indolebutyric acid (IBA; 0-10 µM), pH 5.75 and solidified with 0.25% gellam gum and autoclaved. The shoot explants were cut 2-3 mm below the node region with a scalpel and the leaves above the bottom node removed. Root and shoot regeneration and root length were recorded after 6 w of culture.

Bioreactor Production Systems

Regenerated shoots (Stage 2) and rooted shoots (Stage 3) were transferred to a temporary immersion, thin film nutrient solution bioreactor system for determination of optimal methods of acclimatization. For these experiments, 0.5-1 cm-long shoot explants with a single intact node were cultured in a Liquid Lab™ Rocker system (Southern Sun Biosystems Inc., Hodges, South Carolina, USA). A total of 15 explants were placed in each Liquid Lab™ Vessel (approximately 2.6 l) containing 100 ml of the liquid MS medium devoid of growth regulators or supplemented with 5 µM IAA. The culture medium was replenished after 13 w with 100 ml of the same medium and every 3-4 w thereafter. The vessels were placed on the rocker to provide a gentle side-to-side rotation with a 30 s cycle at 1.2-m interval between cycles. The cultures received 16 h photoperiod with an intensity of 25-40 µmol $m^{-2}s^{-1}$ and the temperature of the growth room housing the liquid lab was 20-24°C. The weights of culture vessel, medium and plant tissues were recorded after 6 months.

Stage 4: Acclimatization to Ex Vitro Conditions

Plantlets obtained from the in vitro liquid lab system were used to assess the rate of transplant survival. Plantlets with at least four roots (approx. 5 cm long) were separated from the clusters developed in the liquid culture vessel, washed with tap water and transferred to 4-in. plastic pots containing a soil-less mix (Promix BX, Premier Horticulture Ltd, Quebec City, Quebec, Canada). The pots were placed in a growth chamber set at 24°C and 95% relative humidity which was reduced by 5% every week. After 5 w, they were transplanted into 6-in. pots and placed in the same chamber adjusted to 70% relative humidity for an additional 12 w followed by transfer to 1-gal. pots in the greenhouse. The experiments were arranged in a completely randomized design. Each treatment consisted of at least 25 explants and experiments were repeated twice. Data were analyzed using the Statistical Analysis System (SAS Institute Inc., 2004) and Student-Newman-Keuls means separation test ($P \leq 0.05$).

RESULTS

Micropropagation System

An efficient micropropagation system was developed for two new cultivars, ‘Ulu fiti’ and ‘Pua'a’. Field collection of mature buds was not successful for establishing these cultivars in vitro. Field-collected meristems quickly became brown and mushy, phenolics were apparent in the medium and a white, latex type of exudate was given off by the mature tissues. To avoid these problems, root cuttings were allowed to become dormant then slowly rehydrated with the first buds to break providing the tissues for establishing the cultures (Fig. 1A). Resulting buds were surface sterilized and established in culture on a cytokinin-supplemented medium with relatively good success (Fig. 1B). However, the cytokinin combination that had previously been successful with other cultivars (Murch et al., 2007) did not efficiently induce shoot regeneration of ‘Ulu fiti’ or ‘Pua'a’ and a series of experiments was conducted to investigate other growth regulators. Supplementation of the culture medium with GA_3 was effective for regeneration of both cultivars’ shoots (Table 1; Fig. 1C, 1D). Further efforts to establish rooting of these regenerated shoots compared the efficacy of various auxins. In contrast to the earlier studies with ‘Ma'afala’, ‘Puou’ and ‘Puupuu’ that found indoleacetic acid was most effective for induction of rooting, ‘Ulu fiti’ and ‘Pua'a’ produced more roots and longer root growth in response to indolebutyric acid (IBA). They produced roots on 100% of the regenerated shoots cultured on the MS media supplemented with 5 µM IBA with an average of 7-8 roots per plantlet. The average length of the roots was 6.8 cm for ‘Ulu fiti’ and 4.5 cm for ‘Pua'a’ after 6 w (Fig. 1E, 1F).

Bioreactor Production Systems

The basic function of a bioreactor is to provide a self-contained, sterile environment with tight control of growth microenvironment. The bioreactor technology is designed for intensive culture of cells, organs and whole plantlets, and is regarded as a key factor for large-scale propagation of cells, organs and whole plants for the production of natural phytochemicals and transgenic proteins. A bioreactor system can produce unlimited volumes of cultured plant material in a short time and also facilitate assessment of plant performance in different growth systems. We have previously established large-scale breadfruit production systems in Liquid Lab™ culture vessels (Murch et al., 2007). In the Liquid Lab™, gentle movement of the vessels with a 30-s cycle and 1.2-m interval between cycles results in contact of the shoots with the liquid nutrient medium in an intermittent microhydrophonic ebb-and-flow system, thereby creating a miniature temporary immersion bioreactor by transferring liquid medium from side-to-side. Shoots begin to form roots within 2 w after subculture into the liquid lab systems and continued culture results in profuse rooting after 8 w (Fig. 1G). Shoots grown in the MS medium devoid of growth regulators developed roots at a rate of 60% while shoots grown in auxin-supplemented medium showed 100% rooting response. Application of these technologies to breadfruit resulted in an optimized system for large-scale production of plants (Murch et al., 2007). The current capacity of existing bioreactors is about 5,000 plants per batch and an average batch takes about 3 months to mature. We recently distributed 4,000 plants by shipping the culture vessels (Fig. 1G), each containing 100 plants. These plants were healthy with extensive root systems and transplanted to field nursery conditions.

Ex Vitro Transplantation

In vitro grown plantlets in the bioreactor for 6-8 w (Fig. 2A, 2B), with well-formed roots in solid or liquid media, were potted and transferred to a growth chamber with high humidity (95%) for a week. Plants were able to withstand a gradual decline in humidity at the rate of 5% per week until reaching 70%. All plantlets survived following the acclimatization process of gradual decline in relative humidity and developed prolific roots (Fig. 2C). After 4 m of growth in the chamber, plants were transferred to standard greenhouse conditions where all of the plants survived and started to grow new leaves within a week. The plants flourished in the greenhouse conditions (Fig. 2D), continuously formed new leaves and were morphologically similar to original plants.

DISCUSSION

Establishment of a High Efficiency Micropropagation Protocol for Breadfruit

We developed a highly efficient protocol for the establishment and sustained proliferation of breadfruit cultures. In initial studies, protocols for ‘Ma'afala’, ‘Puou’ and ‘Puupuu’ were developed (Murch et al., 2007) and, in the current studies, these protocols were adapted for ‘Ulu fiti’ and ‘Pua'a’. The basic procedure involves five distinct phases following the classical stages of a micropropagation system outlined above. In the case of breadfruit, it was also necessary to develop bioreactor systems to accommodate long distance international transport of large numbers of regenerated plantlets.

The most interesting findings of this study relate to the problems that were encountered in the course of the research. Selection of appropriate explant material was complicated by the need to harvest tissues from mature trees in a tropical environment. The culture of shoot tips from mature trees of tropical species, including breadfruit, presents extreme challenges, most notably the in vitro establishment and survival of the mature explants (Rouse-Miller and Duncan, 2000). These problems include severe contamination, browning and necrosis within days of culture, inconsistent response of surviving explants and low efficiency of propagation. Thus, propagation protocols are generally species- or even cultivar-specific. Observations of mature breadfruit shoot tips in initial culture process further confirmed these problems (Murch et al., 2007). For two

cultivars, we were able to reduce these problems by culturing new buds released from root cuttings as they transitioned from dormant to active growth. This approach was successful for 'Ulu fiti' but has not yet been successful for several other cultivars and ongoing research is attempting to establish those first meristem cultures for more than 60 different breadfruit cultivars.

Plant morphogenetic development is regulated primarily by endogenous and exogenous phytohormones, nutrient and stress (Skoog and Miller, 1957; Cassells, 2005; Murch and Saxena, 2005). Cao et al. (2006) recently reported that the efficiency of photosynthesis and the activities of the antioxidant enzymes superoxide dismutase, catalase and peroxidase in breadfruit and jackfruit were season dependent. Oxidative environment is known to play an important role in the successful development of explants cultured in vitro (Cassells and Curry, 2001). That such variations are likely to result in the plant- and explant-specific requirements of in vitro development is clearly evident from reported differences of nutritional and hormonal regimens in various micropropagation studies of tropical trees. In general, both culture response and the mode of in vitro development are known to be regulated by the balance of genotype, nutrients and the growth regulating substances (Murch and Saxena, 2005).

The re-establishment of in vitro plants into field conditions following transport requires that stresses be minimized and that there be adequate, but not excessive, nutrient resources for the plants. Ongoing research is underway to provide international collaborators with optimized protocols that can be adapted to local environments in tropical countries around the world.

The importance of breadfruit as a food staple is well established in the Pacific. With increased distribution of the numerous available cultivars and improved methods for growth and production, breadfruit can become a reliable source of nutritious food in many parts of the world.

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Tables

Table 1. The effects of gibberellic acid on shoot multiplication in two breadfruit cultivars.

Cultivar	'Ulu fiti'		'Pua'a'	
Medium	AS[1]	AS + GA_3[2]	AS[1]	AS + GA_3[2]
Average number of shoots	4.3	7.8	5.0	8.24
Average longest shoot length	3.35	4.31	3	3.95
Average node number	5.53	5.93	4	6.52

[1] *Artocarpus* shooting medium containing 2 µM BA and 3 µM kinetin.

[2] *Artocarpus* shooting medium containing 2 µM BA, 3 µM kinetin and 1 µM GA_3.

Figures

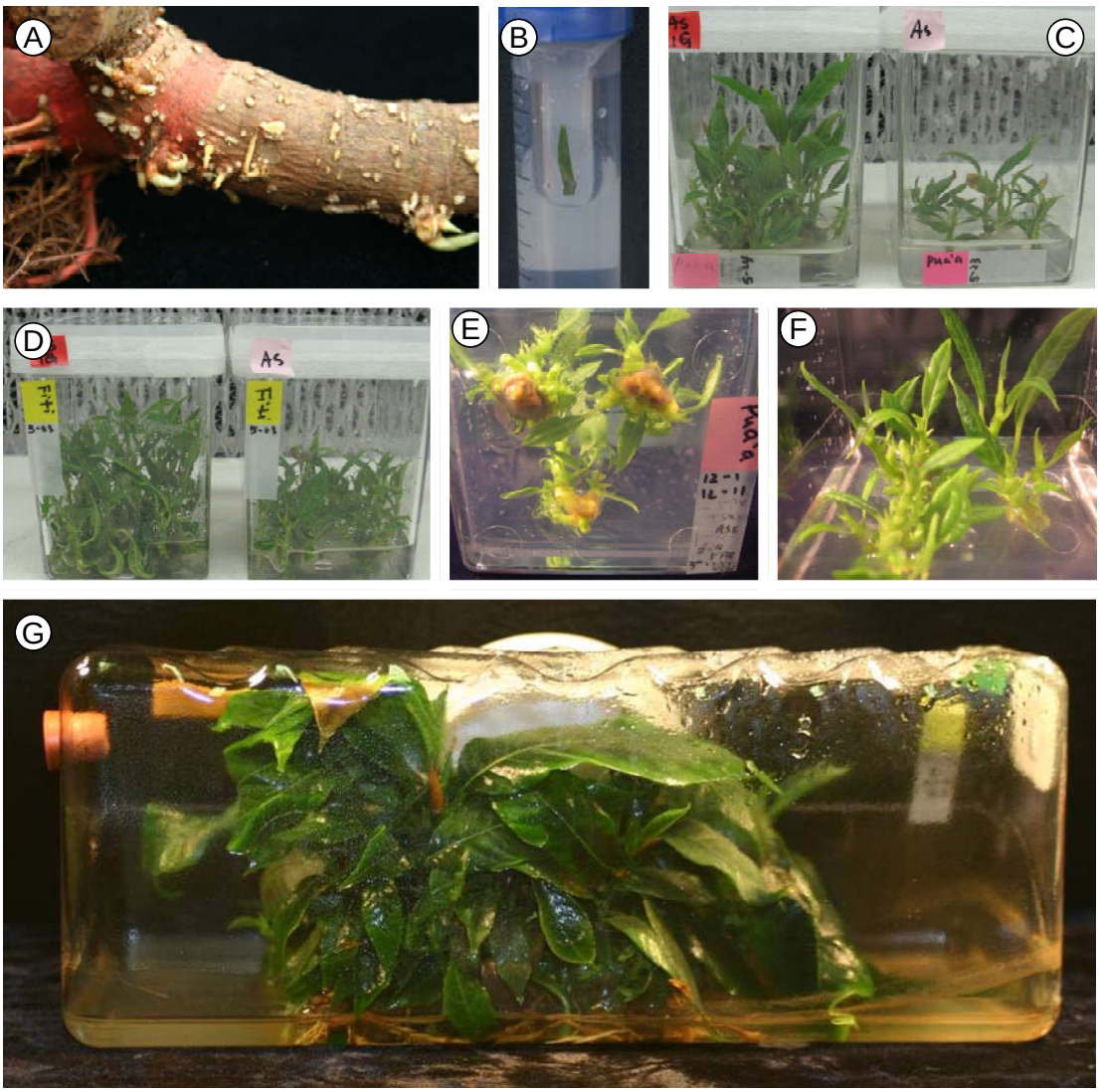

Fig. 1. Development of a micropropagation system for breadfruit (*Artocarpus altilis*) cultivars 'Ulu fiti' and 'Pua'a'. (A) Budbreak from dormant root cuttings was the source of explant material for establishment of initial cultures. (B) Meristem buds were surface sterilized and established in AS medium containing 2 µM BA and 3 µM kinetin. (C) Supplementation of the AS medium with 1 µM GA_3 (left) improved the efficiency of shoot regeneration over the response observed on the AS medium alone (right) in the cultivar 'Pua'a'. (D) The same effect of GA_3 with AS medium (left) was observed with the cultivar 'Ulu fiti'. Root development in 'Pua'a' (E) and 'Ulu fiti' (F) was improved by addition of 5 µM indolebutyric acid. (G) Liquid Lab™ temporary immersion bioreactors were used for large-scale in vitro production and distribution of breadfruit.

Fig. 2. Acclimatization of in vitro grown breadfruit. (A) More than 100 individual breadfruit plants can be grown in each bioreactor vessel. (B) Plants are grown to relative maturity in vitro with well established root systems. (C) In vitro grown plants were established in soil medium in a growth chamber mimicking a tropical environment. (D) Transfer to greenhouse conditions allowed for growth of whole plants.