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Original Article

Isolation and culture of protoplasts of Ma-phut (*Garcinia dulcis*) derived from cell suspension culture

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Abstract

Friable callus induced from young leaves of Ma-phut on Murashige and Skoog (MS) medium containing 3% sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l benzyladenine (BA) and 500 mg/l polyvinylpyrrolidone (PVP), was cultured in liquid medium with the same components. Various ages of cell suspension at weekly intervals were then incubated in various kinds and concentrations of cell wall digestion enzymes combined with 1% macerozyme R-10 on a rotary shaker at 100 rpm under 1500 lux illumination at 26±4°C. Purified protoplasts were cultured at various densities in MS medium (adjusted osmoticum to 0.4 M by mannitol) supplemented with 3% sucrose and two types of auxin, 2,4-D and NAA at four concentrations (1, 2, 3 and 4 mg/l) together with 1 mg/l BA. The results revealed that a four-day old cell suspension culture incubated in 2% cellulase Onozuka R-10 (CR10) in combination with 1% macerozyme R-10 gave an optimum result in both yield and viability of protoplasts at $5.7x10^6$/1 ml PCV and 80%, respectively. Embedding protoplasts at a density of $2.5x10^5$/ml in 0.2% phytagel containing MS medium supplemented with 3 mg/l NAA and 1 mg/l BA promoted the most effective division of the protoplasts (20%). The first division of the protoplasts was obtained after 2 days of culture and further divisions to form micro- and macro-colonies could be observed after 7-10 days of culture. However, callus formation and plantlet regeneration was not obtained.

Keywords: Maphut, protoplast, cell suspension culture

1. Introduction

The genus *Garcinia* belongs to Guttiferae and is composed of at least 49 species including 10 unidentified ones. All *Garcinia* species are native to the Malaysia Archipelago (Lim, 1984). The most important and well-known species that is grown for commercial purposes is mangosteen (*Garcinia mangostana* Linn.). Some other related species such as somkhag (*Garcinia atroviridis* Griff.), pawa (*Garcinia speciosa* Wall.), Ma-phut (*Garcinia dulcis* Kurz.), and cha-muang (*Garcinia cowa* Roxb.) are seen to be grown in backyards. Generally, all species, except mangosteen, can adapt well to drought or dry areas since they have good root systems, which distribute both horizontally (soil surface) and vertically (deep beneath the surface). At the early growth stages the species adapted to dry conditions do not need to be shaded, however mangosteen needs intensive care, especially regular watering and shade. Because of these characteristics, the species adapted to dry conditions have been used as rootstocks for mangosteens to plant in dry areas. Genetic transfer of these useful dry-tolerant characters to mangosteen by pollination is impossible due to apomixis. Biotechnological methods can help to solve this problem. A protocol for clonal propagation of mangosteen has been well documented (Te-chato and Lim, 1999; 2000), so improvements of this species through biotechnological means is quite possible.

Protoplast fusion is one method of transferring a target gene (drought resistance) from a related species to

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mangosteen. Of the possible donor species, Ma-phut is one of the related species, which has previously been successfully used in tissue culture, callus induction, and cell suspension culture (Moosikapala and Te-chato, unpublished data). With appropriate manipulation, the use of this tool for improvements of mangosteen for drought tolerance is feasible. Recently, there have been a few reports on protoplast isolation and culture of *Garcinia* spp. (Te-chato, 1997; 1998), including mangosteen (Te-chato, 1998). Moreover, some important factors affecting isolation and culture of protoplasts from closely related species have also been elucidated (Moosikapala and Te-chato, 2000). However, all of those reports used *in vitro* young leaves as an explant source, and they were incubated with different combinations of enzymes. Cell suspension culture should open a wider range of possibilities due to the rapid cell proliferation in a shorter period of time and uniformity of cells in the suspension.

In this present study, we report the establishment of callus, cell suspension culture, and isolation and culture of protoplasts of Ma-phut, as a prelude to studies on fusion processes, which will be forthcoming.

2. Materials and Methods

2.1 Induction of callus and cell suspension culture

Young leaves of Ma-phut were cultured on Murashige and Skoog (MS) medium containing 3% sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg/l benzyladenine (BA) and 500 mg/l polyvinylpyrrolidone (PVP). The calli, initiated after a month of culture, were subcultured at monthly intervals on to fresh medium of the same components 6 times. Friable callus maintained in this medium at 2 weeks after subculture was transferred to liquid medium with the same components. Initially, the suspension cultures were maintained on a rotary shaker at a fixed speed of 100 rpm under 1500 lux illumination, 14 h photoperiod and 26±4°C. Subculturing was routinely carried out at weekly intervals to reduce large aggregates and secondary metabolite production. After maintenance of the suspension culture for 6 months the cells in the suspension culture were subjected to protoplast isolation.

2.2 Enzyme preparation and incubation conditions

Various kinds and concentrations of cell wall digestion enzymes were compared to optimize a high yield of viable protoplasts. These enzymes were combined with macerated enzyme, macerozyme R-10, at 1%. Each combination of the enzyme was dissolved in a solution of 0.4 M mannitol, adjusted to pH 5.6 and sterilized by passing through a Millipore filter with a pore size of 0.45 mm. 10 ml of the enzyme solution was used to incubate 1 ml of packed cell volume (PCV) in a 50 ml Erlenmeyer flask. The cell and enzyme solution was incubated on a rotary shaker at 100 rpm under 1500 lux illumination at 26±4°C in the same way as described earlier for cell suspension culture maintenance.

2.3 Protoplast isolation and culture

After incubation for 2 hours the resulting solution was then passed through a 77-mm nylon mesh to separate protoplasts from cell debris and aggregates. The filtrate containing intact protoplasts was centrifuged at ca. 800 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 10 ml of washing solution consisting of 0.4 M mannitol. This washing sequence was repeated twice. The final protoplast suspension was purified by floating on 21% sucrose. The complete protoplasts at mid-phase were collected and brought to a known volume and aliquots were taken to measure protoplast yield and viability. In the case of culture, the protoplasts were washed twice with washing solution and once with culture media, then embedded in 0.2% phytagel containing in MS medium supplemented with 0.4 M mannitol, 3% sucrose, 1-4 mg/l NAA or 2,4-D and 1 mg/l BA. In a second method the protoplasts were cultured as a thin layer (3-5 ml) in 6 cm sterile plastic Petri dishes.

2.4 Yield and viability determination

Yield of the protoplasts was determined by two different methods, percentage conversion rate and number of protoplasts. The former method involved comparing packed cell volume of released protoplasts in comparison with initial packed cell volume of the suspension cells (usually 1 ml). The latter method involved counting the protoplasts with a hemacytometer. Viability was assessed using fluorescein diacetate (FDA) as a test of membrane integrity and internal diesterase activity. After 10-15 min in 0.01% (w/v) FDA in a culture medium, the protoplasts were observed under ultraviolet or green light using a fluorescein microscope. The viability percentage was calculated as the number of protoplasts fluorescing green per total number of intact protoplasts x 100.

2.5 Age of cell suspension culture determination

In this study, five different ages of suspension culture cells after subculturing were used. The suspension cells at 3, 4, 5, 6 and 7 days after subculturing were collected and exposed to an enzyme solution consisting of 2% cellulase Onozuka RS and 1% macerozyme R-10. An osmoticum of all the enzyme combinations was adjusted to 0.4 M with mannitol. The cells were incubated in the same way as described previously. Then percent conversion rate of the cells to protoplasts, and the number and viability of protoplasts at different periods after subculturing were determined.

2.6 Optimum enzymes determination

A completely randomized design was used with four types of cellulase: cellulase Onozuka RS (CRS), cellulase

Onozuka R-10 (CR10), cellulase from *Trichoderma viride* (CTV), and cellulase fron *Aspergillus niger* (CAN). Each cellulase enzyme at the same concentration of 2% was combined with 1% macerozyme R-10 and 0.1% pectolyase Y-23. An osmoticum of all the enzyme combinations was adjusted to 0.4 M with mannitol. Suspension cells at 4 days after subculture were exposed to all combinations of the enzymes for a total of 2 hours. Yield and viability measurements were taken at the end of the incubation period.

2.7 Phytohormones and culture density determination

Two types of auxin, 2,4-D and NAA, at four concentrations (1, 2, 3 and 4 mg/l) were tested in combination with 1 mg/l BA. The culture medium used was MS supplemented with 3% sucrose and the osmoticum adjusted to 0.4 M with mannitol. Protoplasts at densities of $5x10^5$/ml, which were isolated by using 1% CR10 in combination with 1% macerozyme and 0.1% pectolyase Y-23 from the suspension cells at 4 days after subculturing, were embedded in the above medium containing 0.2% phytagel. In the case of plating density, fresh protoplasts were adjusted to three densities $1x10^5$, $5x10^5$ and $1x10^6$/ml. The adjusted protoplasts were then embedded in a culture medium as mentioned above, supplemented with 3 mg/l NAA and 1 mg/l BA. Plating efficiency, the percentage of isolated protoplasts undergoing division, was estimated after one week of culture. At the time of plating, random fields of protoplasts in agar were marked by etching a circle 1.2 mm in diameter around the area of interest on the plastic Petri dish. The number of protoplasts in each field was counted. After three weeks, the number of dividing protoplasts in each field was determined. The plating efficiency was calculated as the number of dividing protoplasts divided by the number of protoplasts plated x 100.

2.8 Determination of culture methods

Two different culture methods of the protoplasts, thin layer in liquid medium and embedding, were compared. Protoplasts at a density of $5x10^5$/ml were cultured in the medium supplemented with 3% sucrose, 3 mg/l NAA, and 1 mg/l BA. After one week of culture, the plating efficiency of the two methods was compared.

3. Results

3.1 Age of cell suspension culture determination

Protoplasts could be isolated from 3-day to 7-day-old cell suspension cultures. The yield of protoplasts in terms of both conversion rate and number increased markedly from 3 to 6 days after subculturing and dropped at day 7. Although the 6-day old cells in the suspension resulted in the highest yield of protoplasts, the viability of the same cells had decreased severely (more than half of the amount of 4-day old cells) (Figure 1). Accordingly, the 4-day old cell suspensions were selected as the source of protoplasts for further experiments.

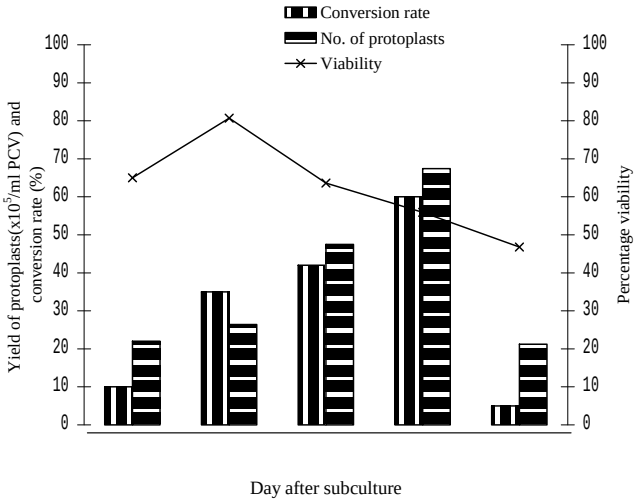

Figure 1. Effect of age of cell suspension after subculture on yield and viability of protoplasts isolated by 1% cellulase Onozuka R10, 1% macerozyme R10 and 0.1% pectolyase Y-23. The enzymes were dissolved in 0.4 M mannitol and pH adjusted to 5.8.

3.2 Optimum enzymes determination

Among the four types of cellulase tested, both 2% of cellulase Onozuka R-10 and RS in combination with 1% macerozyme R-10 and 0.1% pectolyase Y-23 gave a high number of released protoplasts, while the other two enzymes, CTV and CAN, yielded lower quantities. Even though CRS had higher activity than CR10, the number of released protoplasts was far smaller. The number of protoplasts obtained from digestion by CR10 was $5.7x10^6$/1 ml PCV, and from CRS was $2.6x10^6$/1 ml PCV. In the case of viability of the protoplasts, CR10 gave a slightly higher percentage (76.3) than CRS, which gave a survival percentage of 69.1% (Figure 2).

3.3 Phytohormones and culture density determination

Division of the protoplasts was observed after 24 h of culture in all combinations of NAA or 2,4-D with 1 mg/l BA. All concentrations of NAA tested promoted division of the protoplasts better than 2,4-D in all concentrations. NAA at a concentration of 3 mg/l in combination with 1 mg/l BA gave the best results in dividing protoplasts or plating efficiency after a week of culture (12.55%) followed by NAA at concentrations of 2 and 1 mg/l which gave plating efficiencies at 11.7 and 10.9%, respectively. In the case of 2,4-D, 3 mg/l 2,4-D plus 0.5 mg/l BA gave a percentage of protoplast division at 4.6% (Figure 3).

A higher plating density gave a higher number of divisions. The low plating density of $5x10^4$/ml resulted in

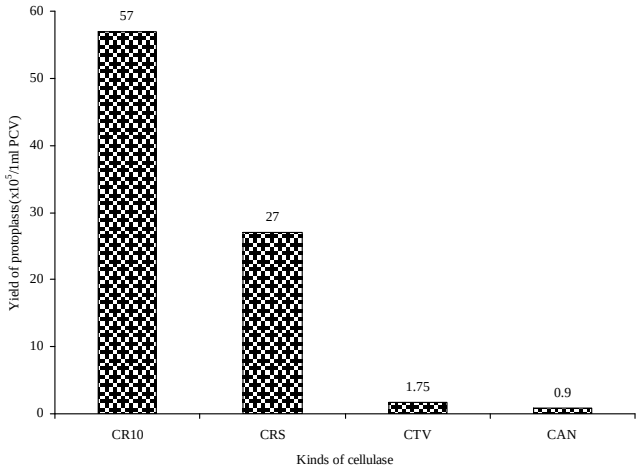

Figure 2. Effect of different cellulase enzymes on protoplast yield. Each kind at 2% was combined with 1% macerozyme R10 and 0.1% pectolyase Y-23
CR10 = cellulase Onozuka R10
CRS = cellulase Onozuka RS
CTV = cellulase from *Trichoderma viride*
CAN = cellulase from *Aspergillus niger*

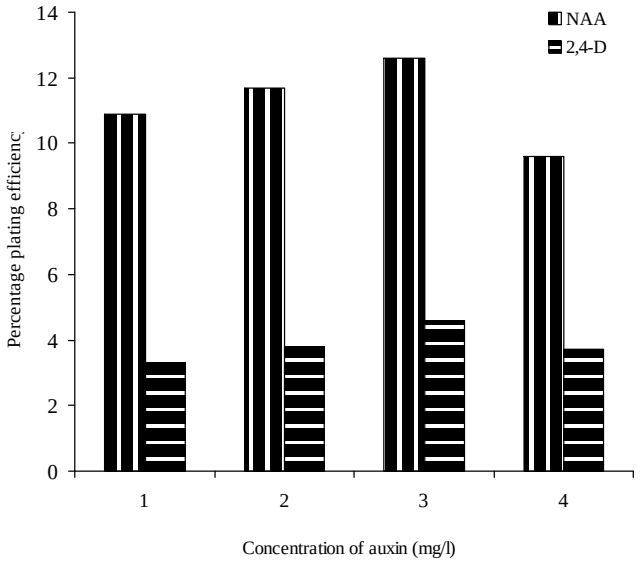

Figure 3. Effect of 2,4-D or NAA at various concentrations on division of the protoplasts when cultured on MS medium supplemented with 0.4M mannitol and 1 mg/l BA at density of $1x10^5$/ml.

the lowest divisions of the protoplasts. A plating density of $2.5x10^5$/ml promoted the highest frequency of divisions of the protoplasts (20%) (Figure 4). All concentrations of NAA tested gave similar results. Increasing the concentration of NAA from 1 to 3 mg/l promoted higher divisions. The maximum number of divided protoplasts was obtained when 3 mg/l NAA was combined with 1 mg/l BA in all plating densities, except $1x10^5$/ml (Figure 4).

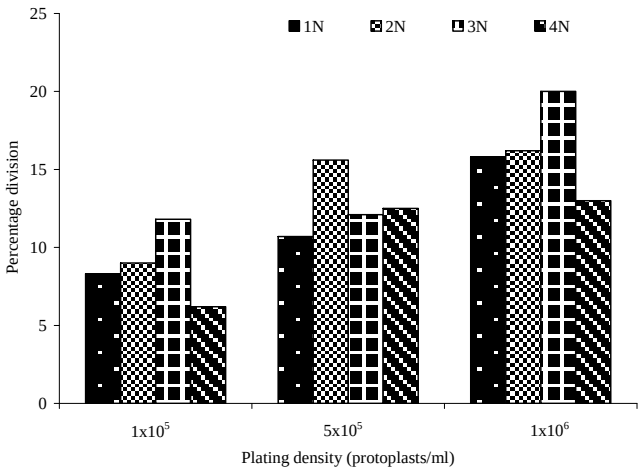

Figure 4. Effect of plating density and concentration of NAA (N) on percentage division of Ma-phut protoplasts cultured on MS medium supplemented with 0.4M mannitol and 1 mg/l BA.

3.4 Determination of culture methods

Embedding the protoplasts in 0.2% phytagel containing MS medium supplemented with all concentrations of NAA and 1 mg/l BA gave a slightly lower result in division of protoplasts than plating in thin layer of liquid medium (Figure 5). NAA at concentration of 3 mg/l in combination with 1 mg/l BA gave the best results in both methods of culture. Culturing by thin layer technique in liquid medium gave the higher division of protoplasts, but symmetric division of the protoplasts was obtained at low percentage. Embedding culture promoted the higher symmetric division.

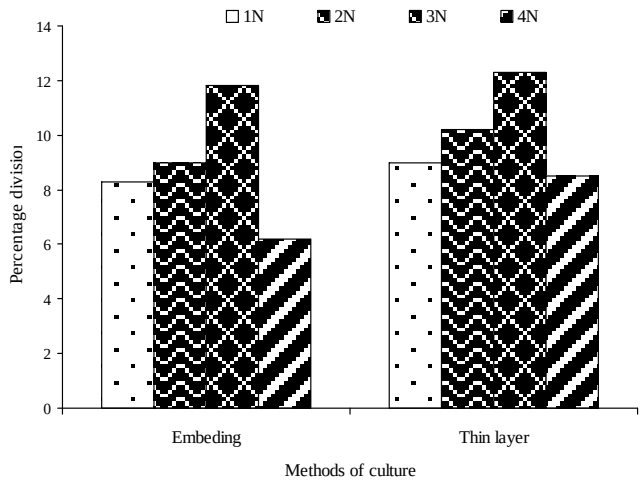

Figure 5. Effect of culturing methods and concentration of NAA on protoplast division of Ma-phut which was cultured on MS medium supplemented with 0.4 M mannitol and 1 mg/l BA and density adjusted to $1x10^6$/ml.

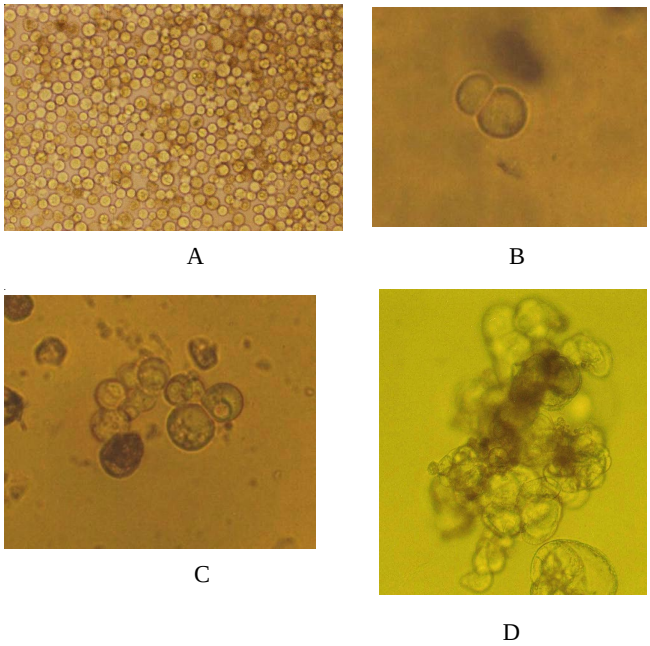

Figure 6. Development of suspension protoplasts in MS medium supplemented with 3% sucrose, 0.4 M mannitol, 3 mg/l NAA and 1 mg/l BA at density of $1x10^6$/ml. (bar = 50 mm)
A: fresh isolated protoplasts
B: first division (1 day of culture)
C: third division (3 days of culture)
D: microcolony formation (10 days of culture)

The first division of the protoplasts from fresh isolate (Figure 6A) obtained in thin layer culture was obtained after 24 hours of culture (Figure 6B). Further divisions in this type of culture were similar to the hypha of fungi (Figure 6C). However, finally, the daughter cells grew to the same size as the mother cells, and each daughter cell started to divide normally to form micro- and macro-colonies after 7-10 days of culture (Figure 6D), while the embedded protoplasts ceased to divide after 8-10 divisions.

4. Discussion

A high yield of viable protoplasts in terms of conversion rate and number of protoplasts could be isolated from a 4-day suspension culture by using 1% cellulase onozuka R-10, 1% macerozyme R-10 and 0.1% pectolyase Y-23. In the case of Somkhag, the same concentration of cellulase R-10 was reported to give the highest yield of protoplasts, but a higher concentration of macerozyme R-10 (2%) was required (Te-chato, 1997). In this present study, 1% macerozyme R-10 was enough to macerate cells from the suspension and to give a large number of protoplasts. Moreover, the number of released protoplasts was improved using pectolyase Y-23 at only a low concentration of 0.1%. For mangosteen, all of those enzymes had been reported to be used at higher concentrations (4% of cellulase Onozuka RS, 2% macerozyme and 1% of pectolyase Y-23) to get a higher yield of protoplasts (Te-chato, 1998). The difference of the current results was mainly due to the different source of the starting material. In the case of mangosteen, leaves were used for isolation of the protoplasts, while suspension cells were used in Ma-phut. The leaves probably have a more complex cell wall composition than cells in suspension. So far, there have been no successful cell suspension cultures of any *Garcinia* except *G. dulcis*. Thus, leaves of those species appear to be a good source for isolation protoplasts. By the use of leaf explants a longer exposure to enzyme solution is required. But for cell suspension culture of Ma-phut, isolation of the protoplasts a lower concentration of mannitol (0.4 M) and a shorter period of time is required. This situation, generally, promoted rapid growth and prolonged division of protoplasts. Division of leaf-derived protoplasts of mangosteen and somkhag was obtained after 2-4 days of culture (Te-chato, 1997; 1998), while suspension cell-derived protoplasts of Ma-phut could be seen in 1 day (24 h). In a suitable culture medium, such as in this present study, macrocolony formation was obtained at 7-10 days of culture. Generally, both auxins and cytokinins promote division and development of protoplasts. The concentration contained in culture media differed greatly from species to species. 2, 4-D has been reported to provide a far greater percentage of divisions of protoplasts than NAA, both in frequency and time consumed (Te-chato, 1997) An adverse effect was obtained in mangosteen (Te-chato, 1998). With auxin, both NAA and 2,4-D played an inhibitory effect in the division rate, whereas TDZ gave a positive result (Te-chato, 1998). In this present study, NAA was superior to 2,4-D and the optimum ratio of NAA:BA was 3:1. Kao and Michayluk (1975) stated that the density of protoplasts in culture media resulted in division and development of them. Optimum density affects the contribution of secondary metabolites produced by one protoplast that stimulates division of another. Hidano and Nuzeki (1988) reported that the optimum density of cultured protoplasts of fruit trees should be between 10^5-10^6/ml. A similar result was obtained in this study. A plating density of 10^6/ml provided the highest division. However, Te-chato (2000) reported that mesophyll protoplasts of Somkhag and mangosteen required a lower plating density ($0.5x10^5$/ml). Usually, success in a protoplast-to-plant system in fruit trees is obtained from an embedded culture (Tamura *et al.*, 1996; Ochatt and Power, 1998a,b,c). In this study, the same results of protoplast division was obtained in both embedding and thin layer culture. Thin layer culture gave slightly better results, but callus and plant regeneration was not seen. A study on the modification of culture techniques, e.g. bead or disc in agarose or phytagel, encapsulated in algenate, needs to be carried out in order to find the best way to use protoplasts as base approaches in somatic hybridization technology and genetically-manipulated mangosteen or related species.

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