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A SIMPLE AND EFFICIENT METHOD FOR ISOLATION OF PINEAPPLE PROTOPLASTS

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ABSTRACT

Protoplasts have showed great value for new germplasms generation. A method was described for the isolation of large numbers of pineapple protoplasts. The procedure utilized 5% mannitol solution as the primary osmoticum and an enzyme mixture of 1.5% macerozyme R-10, 1.5% cellulase R-10 and 5% mannitol solution, pH 5.5, followed by differential and gradient centrifugations. Overall, the method was found to be a simplified and effective alternative to those previously described for pineapple protoplast isolation, obtaining the highest protoplast yield (3.6 × 10⁶/g FW) and the highest protoplast viability (88.9%).

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Introduction

Pineapple (*Ananas Comosus* (L). Merr.) is one of the most famous tropical fruits. Since the beginning of the twentieth century, pineapple breeding has undergone significant efforts in research and development although results have seemed disappointing as the main varieties are still the same as those selected by native Americans before Christopher Columbus' voyages (3). Hybrid breeding and clonal selection are the main conventional breeding methods. However, its self-incompatibility hampered hybrid breeding and clonal selection is tedious work and not a creative method (1). New techniques must be developed to improve pineapple's traits.

Recently protoplast culture and fusion have been successfully used to create new germplasms in many species such as grapevine (2), Petunia and Calibrachoa (4) and *Lupinus angustifoliu*s and *L. subcarnosus* (6). Zhang (7) have preliminarily studied the protoplast isolation of pineapple by pretreating *in vitro* leaves with 0.7 M mannitol for 2.5 h and digested by 0.5% pectinase, 1.0% Cellulase and 2.5% KCl and $MgSO_4 7H_2O$ as osmotic pressure stabilizing agent, pH 5.6~6.0. Pinho Guedes et al. (5) have also isolated protoplast of pineapple by enzymolysis using 2.0% meicelase, 2.0% rhozyme and 0.3% macerozyme R-10 supplemented with 400 mg/L ampicilin, 10 mg/L gentamycin and 10 mg/L tetracycline. However, the above isolation methods are ralatively complicated. In present study *Ananas Comosus* (L). Merr. cv. 'Josapine', the world's first commercial pineapple hybrid, was used as experimental materials and enzymolysis was applied in order to develop a simple and high-yield method for protoplast isolation of pineapple and then could be used to protoplast fusion for improving pineapple.

Materials and Methods

Materials

The lateral buds of ripening fruit of cv. 'Josapine' were used to establish proliferating shoot cultures in Institute of South Subtropical Crops, Chinese Academy of Tropical Agricultural Science. *In vitro* seedlings were subcultured every 6 weeks on Murashige and Skoog (MS) medium containing 1.0 mg/L 6-BA, and pH adjusted to 5.6-5.7. Growing conditions for development of seedlings were 25°C and a 16 h photoperiod. The leaves of shoots, approximately 3.0 cm long, were used for isolating the protoplasts.

Selection of osmotic pressure stabilizing agent

According to previous studies (5, 7), mannitol and sucrose were used as osmotic pressure stabilizing agent. Mannitol concentration gradients were as follows: 5%, 9% and 13%. Sucrose concentration gradients were as follows: 15%, 21%, 27%, 33% and 39%. Leaves were cut into small strips (about 1 mm) and incubated in a 6 cm-width petri dish for 1 h with 10 mL of the above different concentration of mannitol and sucrose. Cell plasmolysis was observed under a microscope. All incubations were carried out at 28°C in the dark on a shaker (40 r/min).

Enzymolysis

Approximately 3 g of leaves were cut into small strips (about 1 mm). Small strips were pre-plasmolysed for 1 h in 10 mL 5% mannitol solution, which was selected as the most suitable osmotic pressure stabilizing agent, and adjusted to pH 5.5. After removing mannitol solution, strips were digested in a 6 cm-width petri dish with 10 mL filter-sterilized (0.2 μm membrane) enzyme mixture of different concentration of macerozyme R-10, Cellulase R-10 and 5% mannitol solution. The condition of enzymolysis was same as that of pre-plasmolyse: 28°C in the dark on a shaker (40 r/min). Isolated

protoplasts were observed with a one-hour interval until the number of protoplasts remained unchanged. The enzyme combinations were shown in **Table 1**.

TABLE 1

Six enzyme combinations

	I	II	III	IV	V	VI
Cellulase R-10 (%)	1.0	1.0	1.0	1.5	1.5	1.5
Macerozyme R-10 (%)	0.5	1.0	1.5	1.5	2.0	3.0

Purification of protoplasts

Protoplasts were released by squeezing the digested tissues and filtering the resulting enzyme-protoplast mixture through a 425 μm mesh. The filtrates were centrifuged at 500 rpm for 10 min. Floating solution was discarded and the pellet of protoplasts was resuspended in 5 mL CPW 27S solution (Cell-protoplast washing solution, containing 27% sucrose). The protoplasts were recovered through repeated centrifugation at 500 rpm for 3 min. Protoplasts were accumulated at the interface of CPW 27S, forming a ring, and were then collected using a pipette. Protoplasts at this stage were allowed to equilibrate in CPW 27S in dark for about 30 min.

Estimation of protoplast yield and viability

Protoplast yield was estimated using a hemocytometer and the data were expressed as yield per gram fresh weight of leaf tissue. The use of the hemocytometer was as follows: a cover was covered in the chamber of the hemocytometer. A drop of protoplast suspension was dropped on the side of the cover. The chamber was slowly filled with protoplast suspension. Under the microscope the number of protoplasts in the visual field was counted.

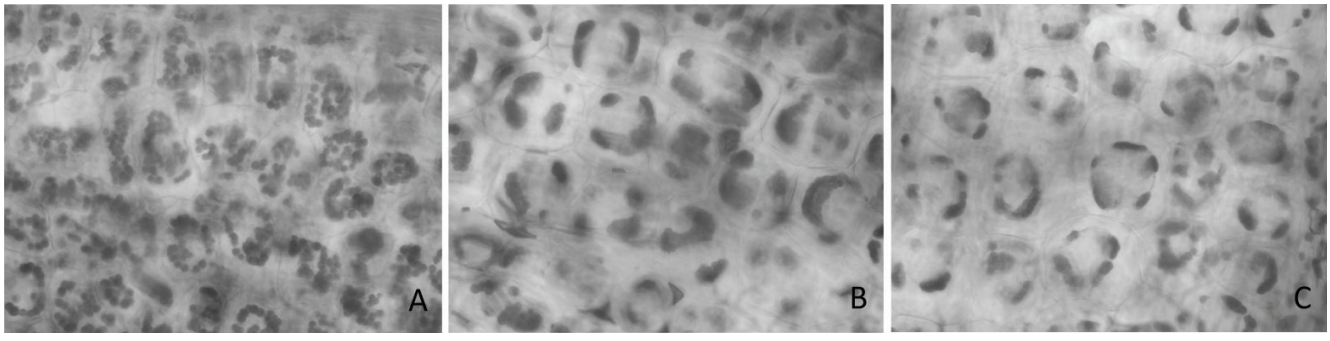

Fig. 1. Plasmolysis under different concentration gradients of mannitol
A: 5%; **B**: 9%; **C**: 13%

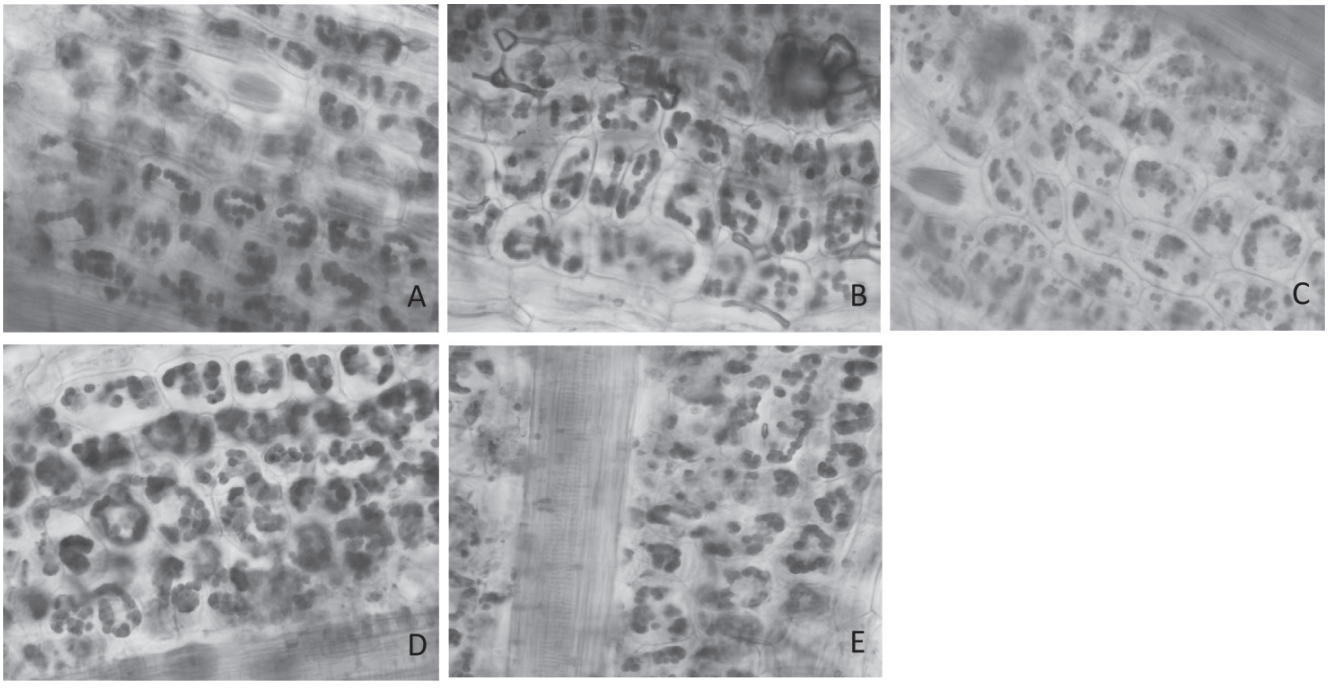

Fig. 2. Plasmolysis under different concentration gradients of sucrose
A: 15%; **B**: 21%; **C**: 27%; **D**: 33%; **E**: 39%

Protoplast viability was determined after fluorescein diacetate (FDA) staining. FDA was added to protoplast suspension at a final concentration at 25 μL FDA per mL protoplast suspension. Protoplasts showing bright fluorescence after 5 min incubation with FDA were counted as viable under fluorescence microscope. Ten visual fields were analyzed and in each visual field the number of protoplasts showing bright fluorescence was counted.

Results and Discussion

Plant cell wall can stabilize intracellular environment and maintain cell survival. When cell wall is removed, the balance of osmotic pressure is changed between inside and outside of cell and then the plasmalemma is dilated or contracted. So osmotic pressure of enzyme mixture, CPW washing solution and culture medium should be same as that of inside of protoplast. Usually it is helpful that osmotic pressure of the above medium is higher than that of inside of protoplast. Mannitol and sucrose were usually used as osmotic pressure stabilizing agents. **Fig. 1** and **Fig. 2** showed plasmolysis under different concentration gradients of mannitol and sucrose. When mannitol concentration and sucrose concentration were 5% and 27%, respectively, they led to plasmolysis. Before enzymolysis, plasmolysis pretreatment could change the cell physiological status, improve the intensity of cell membrane and then decrease the protoplast damage when enzymes are used. So we pretreated leave strips (about 1 mm) for 1 h using 10 mL 5% mannitol solution for pre-plasmolysed.

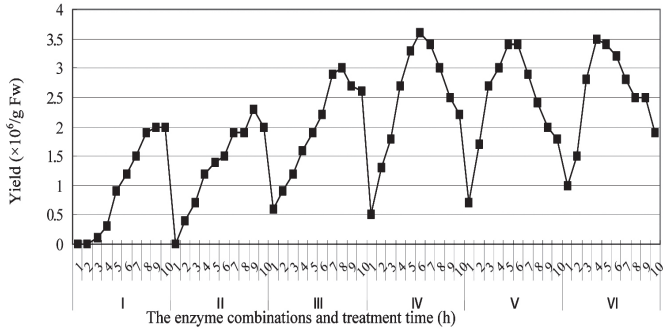

Fig. 3. Effects of different enzyme combinations and treatment time on protoplast yield

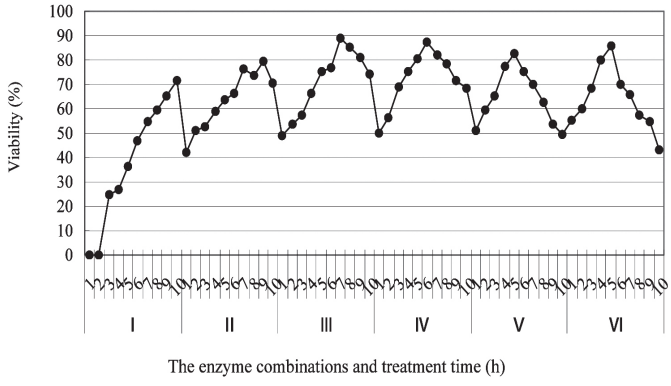

Fig. 4. Effects of different enzyme combinations and treatment time on protoplast viability

Previous studies showed that it was important to choose proper amount and treatment time of enzymes. In our study there were six enzyme mixtures with different concentration. We have also used the concentration of enzyme combination in Zhang' s study (7), meaning enzyme combination I. The result showed that their differences in protoplast yield are not significant in Zhang's and our study. From **Fig. 3**, with increasing concentration of enzymes (macerozyme R-10 and cellulase R-10), protoplast yield was on the rise until it was at the highest peak. Later protoplast yield deceased. When concentration of enzymes was relatively low such as enzyme combination I and II, high protoplast yield required long treatment time. However, long treatment time also led to low protoplast viability using high concentration of enzyme (**Fig. 4**). This could be explained by the toxity of enzymes. The highest protoplast yield (3.6×10^6/g FW) was obtained when using enzyme combination IV for 6 h while the highest protoplast viability (88.9%) was obtained when using enzyme combination III for 7 h. Enzyme combination IV for 6 h also produced higher relatively protoplast viability for 87.5%. **Fig. 5** showed viable protoplasts by FDA staining. Compared with Pinho Guedes et al.'s (5) and Zhang' s study (7), higher protoplast yield and viability would be obtained when using Enzyme combination IV for 6 h. More macerozyme R-10 was used in present study because pineapple leaves are rich in pectin.

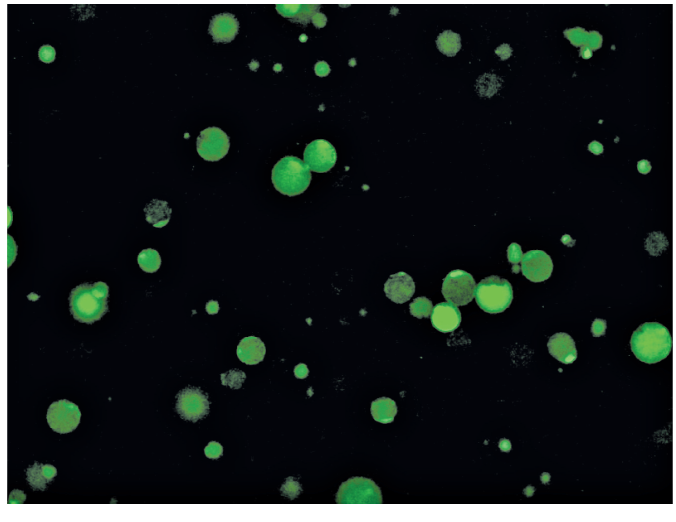

Fig. 5. Viable protoplasts show bright fluorescence after FDA staining

After filtering and centrifuging enzyme-protoplast mixture, CPW washing solution containing 27% sucrose was used for purifying the protoplasts. The purified protoplasts were suspended at the interface of CPW washing solution. In Pinho Guedes et al.' study (5) effective enzymatic mixtures needed six kinds of composition containing meicelase CESB, macerozyme RI, rhozyme HP 150, ampicillin, gentamycin and tetracyline and the protoplasts were purified through centrifuge three times and three kinds of CPW washing medium. In our study only two enzymes, two centrifugations and one kind of CPW washing solution were used.

Conclusions

Papers dealing with isolating pineapple protoplasts are very scarce. The major merit of our study was not only to obtain higher in respect to yield and viability but also to develop an easy protocol. The easy and high efficient protocol could provide a theoretical basis for protoplast culture and fusion and then a new germplasms may be created.

Acknowledgements

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