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Isolation and binary fusion of taro (*Colocasia esculeneta* (L) Schott.) protoplast: Towards developing somatic hybridization protocol as an alternative to sexual hybridization

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***Collocasia esculenta* (L) Schott. (Taro) genotypes cultivated in Kenya rarely produce flowers, thus improvement via pollination is hindered. Somatic hybridization is an attractive alternative to circumvent flower pollination constrain and hence explored in this study. The aim of this study was to optimize isolation and binary fusion of protoplast obtained from *C. esculenta* (Dasheen) and *C. antiquorum* Eddoe) genotypes. Protoplast was isolated from embryogenic calli and leaf tissues using cellulase R-10 (1.0% w/v) combined with pectinase R-10 (0.15% w/v). Fusion of leaf- and calli-derived protoplast was conducted using PEG 6000 (0-30% w/v), $CaCl_2$ (0-0.15 M) and $NaNO_3$ (0-4% w/v). Overall, 2 to 4 h enzyme incubation and PEG (10 or 20%) treatments for 10 to 20 min, were optimal for isolation of viable protoplast and fusion, respectively. The capacity of binary fusions to form cell colonies was higher when fusion was undertaken using PEG at either 10 or 20% after 10 and 20 min incubation. The study demonstrates that optimized protoplast fusion is a viable alternative for taro improvement that by passes flowering constrain.**

Key words: Taro, protoplast, binary fusion, somatic hybridization, PEG 6000, heterokarya.

INTRODUCTION

Taro (*Colocasia esculenta* and *Colocasia antiquorum* L. Schott) is an important staple root crop in Asia, Pacific Islands, and sub-Saharan Africa (SSA) (Chaïr et al., 2016; Grimaldi and van Andel, 2018; Oladimeji et al., 2022). It belongs to family Araceae that comprises at least 100 genera and more than 1500 species (Henriquez et al., 2014). In eastern Africa, taro is cultivated for its edible corms, with production estimated at 2,998,780

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tons in the year 2020 (FAOSTAT, 2021). Taro landrace and cultivars grown in Kenya rarely flower, therefore, development of new varieties or improvement of existing cultivars through pollination is difficult. Dasheen type, locally known as Nduma ngirigacha is the widely cultivated variety in Kenya, though introduced by the colonial government in the 1940s. In addition, three indigenous Dasheen cultivars are either rarely cultivated or grow wildly. These Dasheen cultivars are less popular due to poor yield and high acridity levels (Grimaldi and van Andel, 2018). Indigenous Eddoe type, locally known as Nduma mwanake, grows wildly on farm periphery in central Kenya region (Grimaldi and van Andel, 2018). Despite wider applications of *in vitro* cell culture techniques, there are no reports on protoplast isolation, culture and fusion for taro cultivars from SSA, unlike for pacific and Asia (Verma and Cho, 2010).

Natural hybridization constraints such as parental or genotype incompatibility and flowering difficulty can be addressed via inter specific and inter generic protoplast fusion (Millam et al., 1995; Ranaware et al., 2023). This is because there are few barriers to protoplast fusion and therefore gene transfer can be achieved despite natural hybridization constraints. Somatic hybrids plants are regenerated through embryogenesis or organogenesis. Plant protoplast fusion has successfully been used to transfer disease resistance and other qualitative traits (Dutt et al., 2021). Indeed, availability of reproducible protoplast isolation, fusion and plant regeneration protocols will open new opportunities for developing new or improving SSA taro cultivars. However, protocol development is highly influenced by genotype, donor tissue, enzyme combination, culture media, and physiological status of the cells among others (Reed and Bargmann, 2021; Ranaware et al., 2023). This necessitates optimization or development of suitable protocol for each plant genotype. Therefore, the aim of the study was to optimize isolation and binary fusion of leaf- and calli-derived protoplast obtained from *C. esculenta* (Dasheen) and *C. antiquorum* (Eddoe) genotypes.

MATERIALS AND METHODS

Plant

In vitro stock plants were established using taro cultivar Ngirigacha (Dasheen) and Nduma mwanake (Eddoe) explants collected from farms in Kiambu and Meru counties in Central Kenya Region. Leaf explants were obtained from *in vitro* grown *C. esculenta* and *C. antiquorum* stock plants. On the other hand, embryogenic calli was generated using microcorm (ca. 0.5 mm) slices obtained from *in vitro* stock plants and 1/2 Murashige and Skoog (MS) + 4 mg/L 2,4-Dichlorophenoxyacetic acid (2, 4-D) + 0.5 thidiazuron (TDZ). However, optimal induction of embryogenic calli was first determined using micro-corm explants cultured on semi solid 1/2 MS media containing 2, 4-D (1, 2 and 4 mg/L) alone or combined with TDZ (0.5 or 1 mg/L) in dark at 27°C for 30 days. Embryogenic calli were excised from explants and sub-cultured on respective induction media for 30 days. Media pH was adjusted to 5.8 and was supplemented with sucrose (30 g/L) and gelrite (3 g/L). The number of explants forming calli, type of calli, size of calli and quality proliferating of calli after subculture were assessed.

Protoplast isolation from leaf and embryogenic calli tissues

Protoplast was isolated from leaf and embryogenic calli tissues. Leaves were obtained from *in vitro* grown *C. esculenta* and *C. antiquorum*, while microcorm-embryogenic calli were induced using 1/2 MS+ 2 mg/L 2,4-D + 0.5 mg/L TDZ media. Sliced leaf (ca. 3-4 mm^2) and calli tissues were incubated in dark at 26°C for 1, 2, 4 and 6 h in 5 mL in enzyme cocktail consisting of 0.5% w/v pectinase and 1% w/v cellulose. At the end of each incubation period, tissues-enzyme mixtures were transferred to a sterile nylon mesh (75 µm), then washed using 15% sucrose, pH 5.8 (washing medium). The filtrates were centrifuged at 100 rpm for 5 min, pellets re-suspended in 5 mL washing medium and protoplast fractions recovered using Pasteur pipette. Viable protoplasts for each taro type, explant type, and incubation period were evaluated using the Evan's blue method. The viable protoplasts were adjusted to 4.5×10^6 protoplast/mL.

Screening of chemical fusogens

Fusogens, (i) Polyethylene glycol (PEG) 6000 at 0, 10, 20 and 30% w/v, (ii) $CaCl_2$ at 0, 0.05, 0.1 and 0.15 M, and (iii) $NaNO_3$ at 0, 1, 2 and 4% w/v were screened. PEG and $NaNO_3$ fusogen solutions were supplemented with 0.8 M mannitol and 0.2 M 2-(N-morpholino) ethanesulfonic acid (MES), respectively, while $CaCl_2$ contained 0.4% glycine and 0.8 M mannitol and pH adjusted to 5.8. For fusion, equal volume (200 µL) of leaf and calli protoplast was mixed and then 300 µl of fusogen solutions added dropwise with gentle agitation. Protoplast-fusogen mixtures were incubated at room temperature for 10, 20 and 30 min. Aliquots (50 µL) of fusion products were observed under a microscope (×1000) and occurrence of binary fusions expressed as percentages. Binary fusion products were centrifuged at 500 rpm for 5 min and, pellet re-suspended in 1 mL 1/2 MS+ 2 mg/L 2,4-D + 0.5 mg/L TDZ + 100 mg/L glutamine and transferred into cell culture microplates. The plates were incubated with gentle agitation for 45 days in dark at 25°C to allow formation of micro-calli.

Experimental design and data analysis

All the experiments conducted were laid out in Completely Randomized Design (CRD). For induction of embryogenic calli, the effects of 2, 4-D alone or combined with TDZ were conducted separately as 2×2×3 and 2×2×6 factorial experiments. Three independent replicate experiments were conducted with 15 to 20 explants per treatment. Protoplast isolation and fusion experiments were conducted as 2×2×4 and 3×4 factorial experiments, respectively with 3 replicates per treatment. Data on number of explants forming calli was subjected to square root transformation, while percentage viable protoplast and binary fusion frequencies were transformed using arcsine prior to ANOVA ($p<0.05$) using PROC GLM code of SAS (Version 9.1). The means of treatments were separated using Tukey's Honestly Significant Difference Test ($p<0.05$).

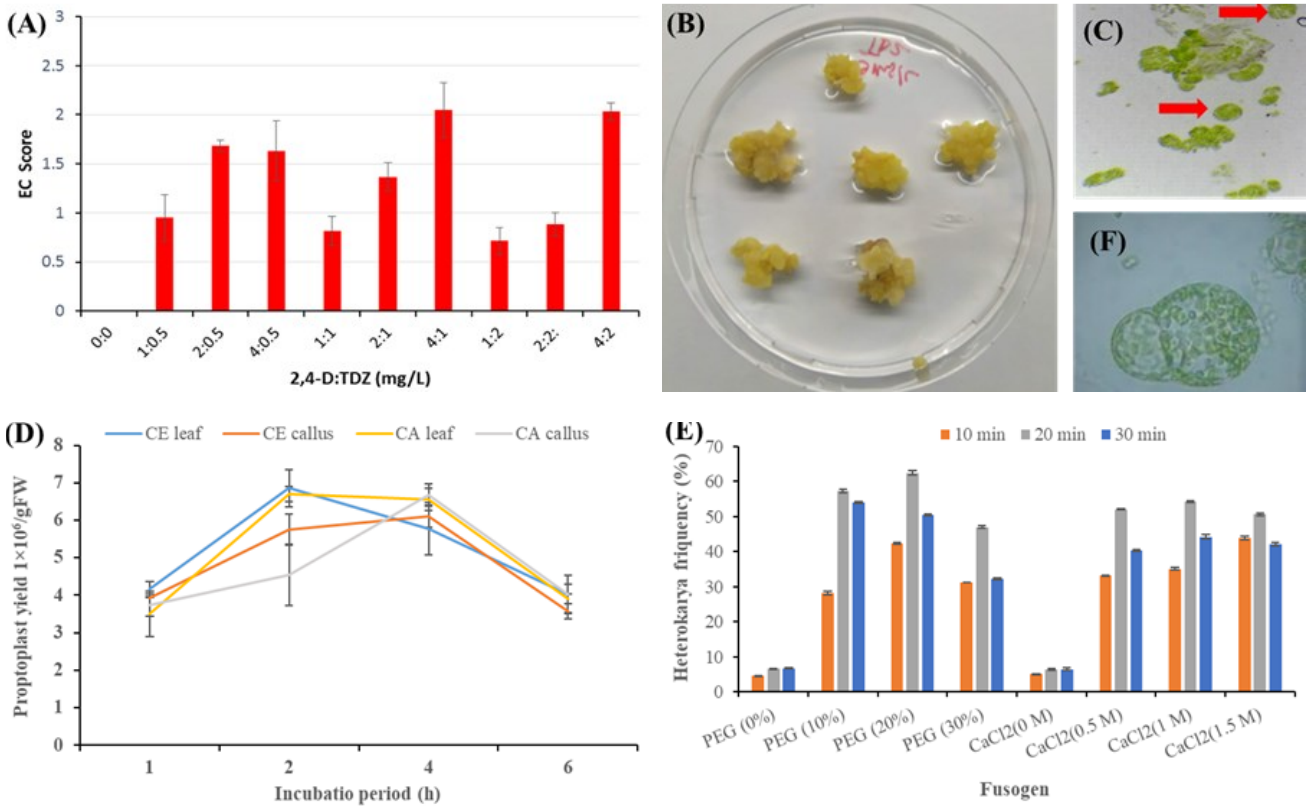

Figure 1. Induction of embryogenic calli, protoplast isolation and binary fusion of leaf- and embryogenic calli-derived protoplast obtained from taro genotypes *Collocasia esculenta* (CE) and *Collocasia esculenta antiquorum* (CA). (A) Quality of embryogenic calli on 1/2 MS media containing different combination of 2, 4-D and TDZ; (B) Proliferation of embryogenic calli on 1/2 MS+ 2, 4-D (4 mg/L) + TDZ (1.0 mg/L) at 30 days after subculture; (C) Protoplast isolated from leaf tissue with red arrow indicating intact protoplast (×1000); (D) Influence of taro genotype, tissue type and enzyme incubation period on protoplast isolation; (E). Determination of best fusogen type and optimal concentration for binary fusion (leaf and calli) of taro protoplast. Graphs are based on the means of 3 independent replicate experiments. Bars on the graphs represent standard error of mean (SEM).

RESULTS

Generally, higher numbers of micro-corm explants from *C. esculenta* and *C. antiquorum* with embryogenic calli were obtained on 1/2 MS media containing 2, 4-D (2 mg/L) combined with TDZ (0.5 mg/L) and 2, 4-D (4 mg/L) combined with TDZ either at 0.5, 1.0 or 2.0 mg/L, unlike 2, 4-D alone, which induced non embrygenic calli. A similar trend was observed on the quality of embryogenic calli induced (Figure 1A). Embryogenic calli exercised from explants proliferated well when sub-cultured on 4 mg/L 2, 4-D + 0.5 or 1 mg/L TDZ (Figure 1B). Leaf and calli derived protoplasts were green (Figure 1C) and transparent to light green in color, respectively. Incomplete release of protoplast from cell walls was observed in all incubation periods. Nonetheless, protoplast yield was influenced ($p<0.05$) by enzyme incubation period and interaction (incubation period × explant tissue). Irrespective of explant type, high numbers of viable protoplast were obtained after 2 and 4 h incubation, for leaf and calli tissues, respectively. However, further incubation > 4 h resulted in yield reduction (Figure 1D). Overall, highest protoplast yield was obtained on leaf explants regardless of taro genotype (Figure 1D). Fusogen concentration, incubation period and interaction (fusogen × incubation) significantly ($p<0.05$) influenced binary fusion frequencies. Regardless of incubation period and concentration, lower (≤33 %) binary fusion frequencies were obtained using $NaNO_3$. On the other hand, 20 min incubation in PEG and $CaCl_2$ across the concentrations tested produced higher binary frequencies, while >20 min incubation reduced fusion frequencies (Figure 1E). Overall, PEG at 20% produced significantly ($p<0.05$) higher (62.5%) number of heterokarya after 20 min incubation (Figure 1E). Majority of the heterokarya observed consisted of leaf- and calli-protoplast fusion. However, fusion of two protoplasts from the same tissue (Figure 1F) and multi-fusions were also

observed. The capacity of binary fusions to form cell colonies was higher when fusion was undertaken using PEG at 10 or 20% after 10 or 20 min incubation.

DISCUSSION

In vitro plant regeneration is influenced by a number of factors; however, for induction of somatic embryos, the key factors are plant growth regulators (PGRs) type, explant type, genotype, and culture conditions (Deo et al., 2010). The results obtained using corm explants from *C. esculenta* and *C. antiquorum*s suggest that genotype was not a major factor that influenced induction of embryogenic calli. Nonetheless, the results demonstrate that PGRs concentration, type and combination had a major influence induction of taro embryogenic calli. These results are in line with studies conducted using Asia Pacific taro genotypes (Deo et al., 2009). Decline in viable protoplast frequency associated with enzyme incubation >4 h for both leaf and calli tissues was not expected. This is because prolonged enzyme incubation is supposed to ensure greater explant penetration and hence increased protoplast yields (Davey et al., 2005). However, the results suggest that prolonged enzyme exposure compromise the integrity of viable protoplasts. Furthermore, protoplast isolation mimics wounding stress in plants and thus enzyme exposure >4 h might have led to acidification of cytosol due to accumulation of oligo-galacto lipids that led to disintegration of isolated protoplasts (Barnes et al., 2019). Therefore, isolation of taro protoplast from leaf and calli tissues was optimal within 2 to 4 h incubation. Despite higher yields obtained from leaf tissues, both explant type and taro genotype influenced viable protoplast yield. The genotype effect on taro protoplast yields has been reported and was ascribed to varied release of compounds from chopped leaves that inhibited enzymatic cell wall digestion (Reed and Bargmann, 2021). Fusogens reduce plasma membrane negative charges; hence, allowing protoplasts to fuse (Ahmed et al., 2021). Therefore, variations in binary fusion frequencies can be attributed to varied effects of fusogen types and concentrations on reducing negative plasma membrane charges. On the other hand, the inverse relationship between binary fusion frequencies and fusogen concentrations obtained, clearly demonstrate that prolonged exposure and higher fusogen concentration did not favor fusion of taro protoplast.

Conclusion

Induction of friable embryogenic calli using micro corm explant was optimal when 24-D (2-4 mg/L) was combined with TDZ (0.5 mg/L). Whereas, 2 to 4 h incubation of leaf and embryogenic calli tissues in cellulase R-10 (1.0% w/v) combined with pectinase R-10 (0.15% w/v) was sufficient for isolation of viable protoplast. On the other hand, PEG (10 or 20%) was optimal for fusing leaf and embryogenic calli protoplast. Protoplast isolation and fusion protocol described in the study provides a simple procedure that ensures satisfactory yields, quick recovery of viable protoplasts, and heterokarya. Protoplast survival and division after fusion ascertained the competence of the protoplasts isolated. However, further research to determine optimal media formulation for culturing taro heterokarya and subsequent plant regeneration is required. The study demonstrates that optimized protoplast fusion coupled with plant regeneration is a viable alternative to natural hybridization of taro via cross-pollination.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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