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Protoplast isolation for species in the *Chamelaucium* group and the effect of antioxidant enzymes (superoxide dismutase and catalase) on protoplast viability

Kanokwan Ratanasanobon · Kevin A. Seaton

Abstract An effective protocol for protoplast isolation from young leaves and somatic embryogenic cells of species in the *Chamelaucium* group and the use of superoxide dismutase (SOD) and catalase (CAT) to enhance protoplast viability are described. Mesophyll protoplasts were isolated from young leaves of a white Geraldton waxflower (*Chamelaucium uncinatum*) line 583, using a mixture of 1% (w/v) cellulase R10, 0.5% (w/v) macerozyme R10, and 0.1% (w/v) pectolyase. Viability of isolated mesophyll protoplasts increased dramatically when SOD and CAT were added. The highest increase of 7.61-fold in viability and 4.34-fold of viable protoplast yield were achieved when a combination of SOD at 500 units mL^{-1} and CAT at 2,000 units mL^{-1} was added to the enzyme mixture. Somatic embryogenic cell-derived protoplasts were isolated from embryogenic suspension cells of *C. uncinatum* line 583 when 1% (w/v) hemicellulase was added to a combination of 2% (w/v) cellulase R10, and 1% (w/v) macerozyme R10. Addition of SOD at 500 units mL^{-1} and CAT at 2,000 units mL^{-1} to the enzyme mixture improved viability only slightly, to above 90%, but improved yield significantly (6.6-fold). This combination of enzymes was also used to isolate protoplasts from embryogenic suspension cells of *Chamelaucium repens* and from young leaves of *C. uncinatum*, *Actinodium calocephalum*, *Verticordia etheliana*, *Verticordia grandis*, *Verticordia hughanii*, and *Verticordia mitchelliana* successfully with viability >80% and viable yield >7× 10^5 cells g^{-1} fresh weight (or per milliliter packed cell volume in the case of suspension cells).

K. Ratanasanobon (✉) · K. A. Seaton
Irrigated Agriculture Innovation, Irrigated Agriculture and Diversification Division, Department of Agriculture and Food, Western Australia, 3 Baron-Hay Court,
South Perth, WA 6151, Australia
e-mail: kratanasanobon@gmail.com

Introduction

Geraldton waxflower (*Chamelaucium uncinatum* Schauer) is one of the main Australian wildflowers grown for cut flowers and is sold on domestic and international markets. The wildflower production in Australia was estimated at 50 million Australian dollars (wholesale) in 2005 (RIRDC 2008). Although the *Chamelaucium* group [*Chamelaucium* and the related genera in monophyletic group of tribe Chamelaucieae, family Myrtaceae (*Verticordia*, *Darwinia*, *Homoranthus*, *Actinodium*, and *Pileanthus*)] provides a rich genetic resource with 293 taxa recognized (Barrett 2006), using conventional hybridization to breed novel varieties has had limited success. The frequency of hybrids occurring when parents are genetically more distantly related was low with hybrids obtained being sterile (Shan and Seaton 2009). Somatic hybridization is a technique that enables combination of somatic cells (whole or partial) by protoplast fusion. This process will bypass reproductive isolation barriers that occur during conventional hybridization.

An effective protoplast isolation method to obtain high yield and viable protoplasts is a prerequisite for somatic hybridization. Factors that affect protoplast yield and viability are the source of the protoplasts and the type of plant cell wall-digesting enzyme used in the isolation. Generally, different protoplast sources require different enzymes to isolate protoplasts as they have different compositions of intra- and intercellular tissue. For example, different combinations of enzymes have been used to isolate protoplasts from leaves and embryogenic culture cells of *Solanum* species (Tan et al.

1987), citrus (Ohgawara et al. 1991), and mango (Rezazadeh et al. 2011).

Apart from nutrient composition and culture conditions, oxidative stress has been shown to affect protoplast viability and regeneration ability. Oxidative stress due to accumulation of active oxygen species [superoxide anion radical ($O_2{}^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (^-OH)] during enzymatic digestion of plant cell walls has been reported to play an important role in recalcitrance of protoplast regeneration in several plant species including cereals (Cutler et al. 1991), grape (Papadakis and Roubelakis-Angelakis 1999), and Grevilleas (Kennedy and De Filippis 2004). These studies suggested that active oxygen species (AOS) were toxic to the protoplast plasma membrane and subsequently affected the process of cell wall reconstitution, which is the first step of protoplast regeneration. AOS were generated during enzymatic digestion of cell walls (Ishii 1987), especially if commercial macerating enzymes containing xylanase were used during protoplast isolation (Ishii 1988; Papadakis and Roubelakis-Angelakis 1999). AOS were also generated by wounding plant tissues (Cutler et al. 1991; Dat et al. 2000). This suggested that AOS were produced when tissues, such as leaf tissues being used as the protoplast source, were cut or peeled in preparation for maceration with digesting enzymes.

In higher plants, to cope with AOS accumulation during stress, antioxidation strategies are employed. These include production of antioxidant enzymes. The main enzyme is superoxide dismutase (SOD), which dismutates $O_2{}^{\cdot-}$ and produces H_2O_2; in turn, H_2O_2 is eliminated by many enzymes including catalase (CAT) by converting two molecules of H_2O_2 to H_2O and O_2 (Dat et al. 2000). SOD and CAT have also been used to improve viability of enzymatically isolated protoplasts of rice (Ishii 1988).

This research defines an effective protocol for protoplast isolation of species within the *Chamelaucium* group from young leaves and somatic embryogenic cell suspension by using different combinations of cell wall-digesting enzymes. Further, the use of SOD and CAT to enhance protoplast yield and viability was examined.

Materials and Methods

Plant materials and cell suspension cultures. Plant materials used were *C. uncinatum* Schauer (line 583), *Chamelaucium repens* A.S. George, *Actinodium calocephalum* N.G. Marchant, *Verticordia etheliana* C.A. Gardner, *Verticordia grandis* J. Drumm, *Verticordia hughanii* F. Muell, and *Verticordia mitchelliana* C.A. Gardner. Young leaves from these plants used for protoplast isolation were obtained from *in vitro* shoots that had been cultured as described by

Ratanasanobon and Seaton (2010) for at least 12 wk with transfer to fresh medium every 4 wk.

Cell suspension cultures of *C. uncinatum* line 583 and *C. repens* were initiated from somatic embryogenic callus obtained from cultures produced by the method described by Ratanasanobon and Seaton (2010). Two-week-old immature seed-derived somatic embryogenic callus of *C. uncinatum* line 583 and leaf-derived somatic embryogenic callus of *C. repens* were used for cell suspension initiation. For each treatment, 5 g of callus was inoculated into 50 mL Murashige and Skoog basal medium (Murashige and Skoog 1962) supplemented with 0.5 μM 2,4-dichlorophenoxyacetic acid, 20 g L^{-1} sucrose, and 5 mM 4-morpholineethanesulphonic acid at pH 5.8 (MSL05) in a sterile 250-mL Erlenmeyer flask with a cap. The inoculated flasks were placed on a rotary shaker at 120 rpm in the dark at $25\pm1°C$ for 1 wk, after which the suspension cultures were passed through sterile 250-μm mesh filters. The flow-through supernatant was centrifuged at 150 relative centrifugal force; cell aggregates were collected, then resuspended in fresh media and cultured under the same conditions as described above. Suspension cells were subcultured every 2 wk by inoculating 25 mL of the cultured cells into 25 mL of fresh medium. Suspension cells were subcultured at least four times before being used for protoplast isolation.

Protoplast isolation and viability test. The combinations of enzymes in the digestion mixtures are shown in Table 1. The mixture solutions were made up with enzymes [Sigma (Sydney, Australia), except Macerozyme R10 (Karlan, Phoenix, AZ)] and CPW06M solution containing CPW salts (Patat-Ochatt et al. 1988) with 0.6 M mannitol and were adjusted to pH 5.0. SOD derived from bovine erythrocytes was made up to a 10,000 units mL^{-1} stock solution with deionized water. CAT derived from bovine liver was made up to 20,000 units mL^{-1} stock solution with 50 mM potassium phosphate buffer at pH 7. All enzyme solutions were filter-sterilized.

The protoplast isolation process involved plasmolysis, digestion, and purification steps. Half a gram of 2-wk-old leaves of *in vitro* shoots was submerged in 5 mL of CPW06M and was gently bruised with a glass rod then cut into fine pieces. The supernatant was withdrawn and a new 5 mL CPW06M was added. The finely cut leaves were left for plasmolysis at room temperature ($22\pm1°C$) in the dark while being shaken gently (40 rpm) for 1–2 h.

Ten milliliters of 1-wk-old cell suspension was centrifuged at 60 relative centrifugal force (RCF) for 10 min and the pellet of 1 mL packed cell volume (PCV) was resuspended with 10 mL CPW06M. The suspension was then placed in the dark at room temperature ($22\pm1°C$) for 1–2 h for plasmolysis with mixing every 15 min.

After plasmolysis, the solution was replaced with 5 mL enzyme mixture. The digestion mixture was incubated at 25

Table 1. Composition of enzyme mixtures used in protoplast isolation

Enzyme mixture	Amount of enzyme in mixture [% (*w/v*)]			
	Cellulase R10	Macerozyme R10	Pectolyase	Hemicellulase
CM (cellulase R10+macerozyme R10)[a]	2	1	0	0
CMP (cellulase R10+macerozyme R10+pectolyase)[b]	1	0.5	0.1	0
CMH (cellulase R10+macerozyme R10+hemicellulase)[c]	2	1	0	1

[a] From Latif et al. (2002)

[b] From Latif (2003)

[c] From this study

±1°C in the dark with gentle shaking (40 rpm) overnight (16–18 h). After digestion, protoplasts were harvested by two-stage filtering through 250-μm then 45-μm sterile nylon sieves. Protoplasts were washed by centrifuging and resuspending in 4 mL CPW06M. Protoplasts were further purified by floating on 10 mL of CPW solution containing 21% (*w/v*) sucrose (CPW21S) and centrifuging (180 RCF, 10 min) to separate the protoplasts from the cell debris. The purified protoplasts (the band between CPW06M and CPW21S after centrifuging) were resuspended in 2–4 mL CPW06M solution.

The viability of protoplasts was determined by staining with fluorescein diacetate at a final concentration of 0.005% (*w/v*). After 5-min staining, protoplasts were observed under UV and viable protoplasts (fluorescent) were counted. Viability was expressed as the percentage of the number of protoplasts that fluoresced against the total number of protoplasts counted. Protoplast density was determined by using a haemocytometer (Blaubrand®, Wertheim, Germany). Protoplast yield was measured as the total protoplast number per

isolation (protoplast density×volume of protoplasts resuspended in CPW06M after purification). Ten counts were carried out and a mean of viability (in percent) and yield was calculated for each replicate in treatment.

SOD and CAT treatment. Addition of SOD and CAT to the enzyme mixture to enhance protoplast viability was investigated. SOD amounts varied from 0, 300, 500, and 1,000 units mL^{-1} enzyme mixture in combination with 2,000 units mL^{-1} CAT.

Digesting enzymes treatments. Three different formulations of enzyme mixture with or without SOD and CAT were tested (Table 1). The best isolation solution mixture was then tested for its effectiveness with other species within the *Chamelaucium* group, which were *C. repens, A. calocephalum, V. etheliana, V. grandis, V. hughanii,* and *V. mitchelliana.*

Statistical analysis Data was collected from experiments with three replicates per treatment. Statistical analyses were

Figure 1. Effect of SOD and CAT on percent viability, total yield, and viable yield of mesophyll protoplasts isolated from *C. uncinatum* line 583 leaves with CMP enzyme mixture. CAT amount was fixed at 2,000 units mL^{-1}, SOD amount (units per milliliter) is indicated in *brackets*. Means and SE are shown. Treatments with the same *letter* showed significant difference at *P*<0.05 using Tukey's test.
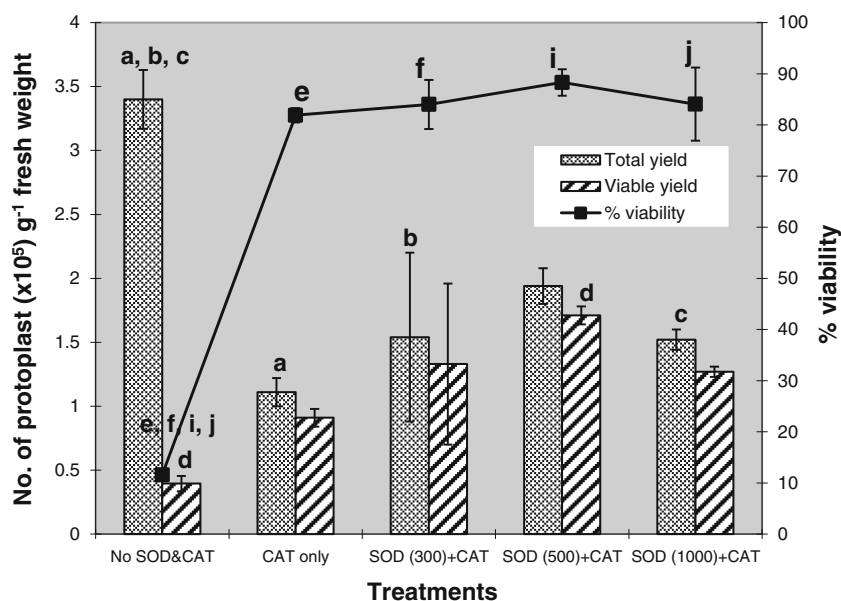
Figure 2. Effect of different formulations of enzyme mixtures (Table 1) on percent viability, total yield, and viable yield of protoplasts isolated from embryogenic suspension cells of *C. uncinatum* (CU) and *C. repens* (CR). SOD at 500 units mL^{-1} and CAT at 2,000 units mL^{-1} were used. Means and SE are shown. Treatments with the same *letter* showed significant differences at *P*<0.05 using Tukey's test.
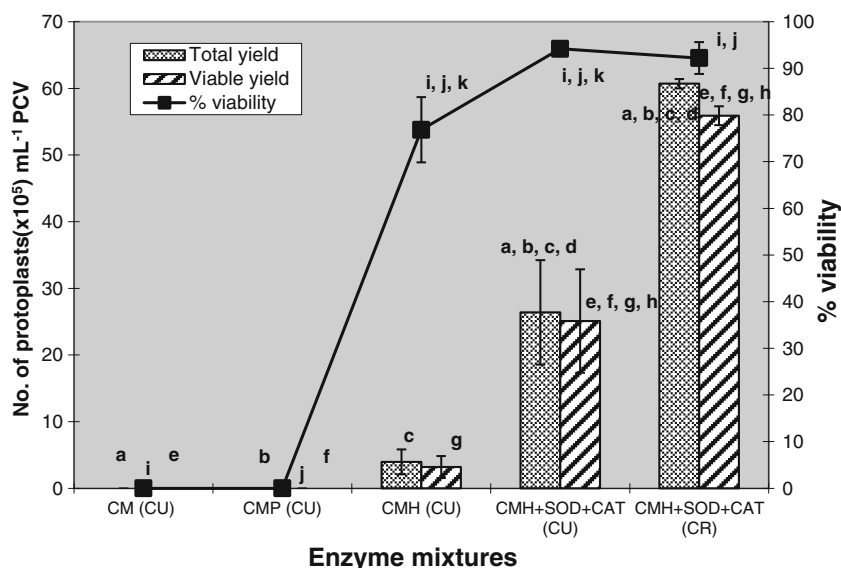

carried out using analysis of variance with Tukey's test for post hoc comparisons at *P*=0.05.

Results and Discussion

The effect of SOD and CAT on mesophyll protoplast viability. Mesophyll protoplasts isolated from *in vitro* young leaves of *C. uncinatum* (line 583) using CMP enzyme mixture (Table 1) without SOD and CAT produced protoplasts with low viability (11.6±1.15%). The addition of SOD and CAT improved viability significantly (*P*<0.05), although it did not improve yield (Fig. 1). The maximum viable yield for mesophyll protoplasts using SOD and CAT in the digestion mixture was found at 500 units mL^{-1} SOD

and 2,000 units mL^{-1} CAT, producing a viability of 88.3± 2.6% and a viable yield of 1.71±0.07×10^5 protoplasts per gram fresh weight. This was an increase of 7.61-fold percent viability and 4.34-fold viable yield compared with digestion without SOD and CAT. This combination of CAT and SOD was used in subsequent experiments. In the preparation of Geraldton wax leaf tissues for enzymatic digestion, it was necessary to bruise leaves gently before cutting them into fine pieces because its leaves are hardy and have a thick cuticle that made enzyme mixture penetration difficult. This wounding procedure may have generated AOS that caused a low percent viability of mesophyll protoplasts isolated. AOS accumulation after mechanical wounding has been reported in many plant species including winter squash and potato (Dat et al. 2000). SOD and CAT are enzymes in the plant

Figure 3. Effect of enzyme combinations on percent viability, total yield, and viable yield for mesophyll protoplast isolation from *C. uncinatum* (CU), *A. calocephalum* (AC), *V. etheliana* (VE), *V. hughanii* (VH), *V. grandis* (VG), and *V. mitchelliana* (VM). SOD at 500 units mL^{-1} and CAT at 2,000 units mL^{-1} were used. Means and SE are shown. Treatments with the same *letter* showed significant differences at *P*<0.05 using Tukey's test.
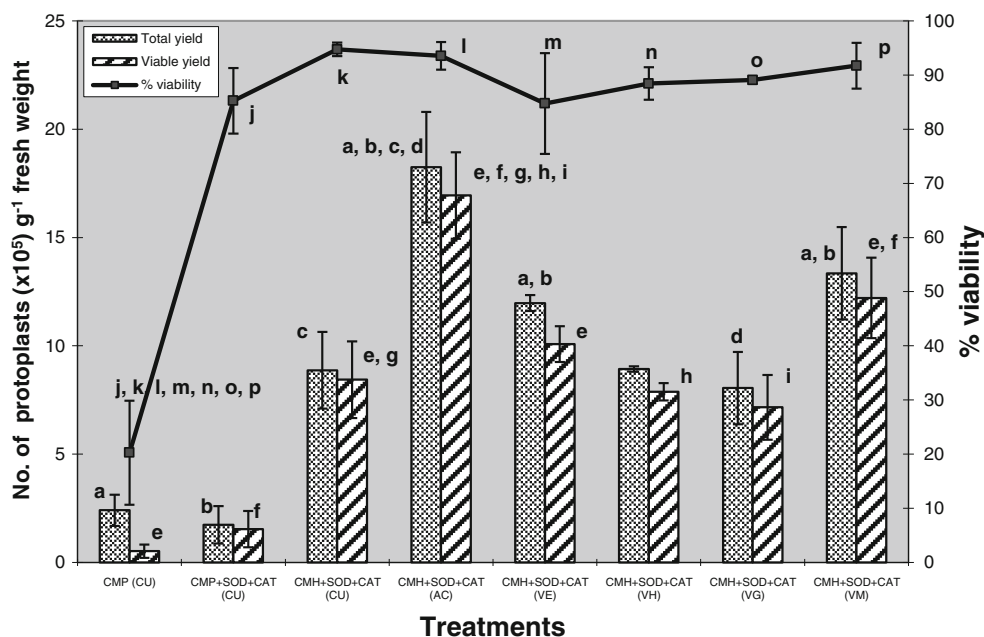
antioxidant system, which together convert toxic AOS to water and oxygen that are not toxic to plant.

Enzyme combination for protoplast isolation from suspension cells. Three combinations of enzymes (Table 1) were used to isolate protoplasts from embryogenic suspension cells. CM and CMP were tested in this study because they were reported to isolate protoplasts successfully from suspension cells of *C. uncinatum* (Latif et al. 2002; Latif 2003). During digestion with CM and CMP enzyme mixtures, cells were partially digested after 6 h and no protoplasts were released. At the end of the digestion period (~16 h), protoplasts were rarely observed. After the purification process, a zero protoplast yield was obtained (Fig. 2). However, protoplasts could be isolated when the CMH mixture was used. A source of suspension cells might be an explanation for the outcomes of using these three enzyme combinations in this trial. Suspension cells which were used in protoplast isolation with CM and CMP in Latif et al. (2002) and Latif (2003) were derived from undifferentiated friable calli (Latif et al. 2002) and they might have different cell wall components from suspension cells that were derived from somatic embryogenic calli which were used in this study. Changes in cell wall components were observed when undifferentiated cells differentiated to embryogenic competent cells (Feher et al. 2003). Macerozyme is a combination of pectinase and hemicellulase. Macerozyme present in CM alone would be expected to achieve sufficient digestion of hemicellulose, which is a component of suspension cells (Bauer et al. 1973; Wilder and Albersheim 1973; Thomas et al. 1987), to release protoplasts from suspension cells but it did not. Protoplasts were produced successfully when hemicellulase was added to the digestion mixture CMH.

The addition of 500 units mL^{-1} SOD and 2,000 units mL^{-1} CAT to CMH improved viability slightly (22.6%) but improved yield significantly (6.6-fold). CMH with 500 units mL^{-1} SOD and 2,000 units mL^{-1} CAT was then used to isolate embryogenic suspension cells of *C. repens* and yielded viable protoplasts at $55.9 \pm 1.42 \times 10^5$ mL^{-1} PCV with $92.2 \pm 3.4\%$ viability.

The use of CMH enzyme mixture with SOD and CAT in mesophyll protoplast isolation of other members in the Chamelaucium group. The use of CMH with 500 units mL^{-1} SOD and 2,000 units mL^{-1} CAT was tested in mesophyll protoplast isolation from *C. uncinatum*. An increase of 16.23- and 5.48-fold in viable yield was achieved compared to CMP and CMP with 500 units mL^{-1} SOD and 2,000 units mL^{-1} CAT, respectively (Fig. 3). Therefore, this enzyme combination was used to isolate mesophyll protoplasts from *A. calocephalum*, *V. etheliana*, *V. grandis*, *V. hughanii*, and *V. mitchelliana*. It successfully released protoplasts with viability >80% and viable yield $>7 \times 10^5$ g^{-1} fresh weight (Fig. 3).

Conclusions

This is the first report of successful protoplast isolation from young leaves of *C. uncinatum* with high viability and viable yield above 8×10^5 g^{-1} fresh weight using the antioxidant enzymes SOD and CAT. The protocol for protoplast isolation from embryogenic suspension cells of *C. uncinatum* also proved successful for efficient and reproducible isolation mesophyll protoplasts from other species in the *Chamelaucium* group.

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