Camelina sativa Protoplast Isolation, Purification, and Culture

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Abstract

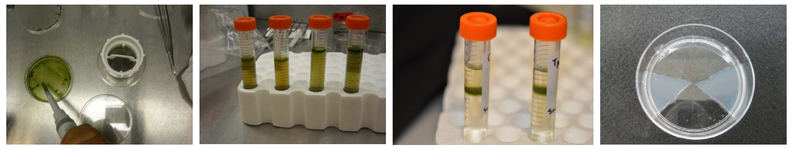
Camelina sativa is a renewable, non-food biofuel crop with a high seed oil content. However, its oil shows high oxidative susceptibility due to its high polyunsaturated fatty acids content. This disadvantage can be overcome through genetic engineering via protoplast transformation. Efficient tissue culture methods and transformation protocols are required for successful transformation. We tested the effect of enzyme concentration, digestion temperature, purification methods, and culture environment on protoplast yield and regeneration in order to refine the steps of protoplast isolation to enable future genetic engineering of *C. sativa*. We achieved the highest yield of viable protoplasts with a 25 ^{o}C incubation of C1M1 enzyme containing mannitol and a washing solution isolation.

Introduction

Biofuels are clean, renewable fuels that derive energy from plant carbon fixation and provide an urgently needed alternative fuel source as global warming increases and oil supplies dwindle. Current research aims to develop non-food crop plants, such as *Camelina sativa,* as biofuel feedstocks. C. sativa grows well in semi-arid conditions, is cold-tolerant, and requires few inputs, permitting growth on marginal lands with low production costs (Canadian Food Inspection Agency, 2012). Its seeds contain 38 to 43% oil, 45% of which is polyunsaturated, and produce 62 gallons of biodiesel/acre, sharply contrasting with corn's 18 gallons/acre (USDA-ARS and WSU Fact Sheet; Leonard, 1998). Unfortunately, the high percentage of polyunsaturated fatty acids in *Camelina* oil causes susceptibility to oxidation, making oil both difficult to store and unattractive for use in large-scale production (Lu &Kang, 2008).

Camelina's oxidative instability, however, can be overcome by introducing foreign gene(s) via genetic engineering. Multiple transformation methods exist, of which protoplast (plant cells without cell walls) transformation is the simplest. For successful protoplast transformation, protocols for protoplast isolation, purification, culture, and regeneration must be optimized. Five groups have successfully isolated *C. sativa* protoplasts in order to improve other *Brassicaceae* species through somatic hybridization (Canadian Food Inspection Agency, 2012). We are isolating protoplasts with the explicit intention of improving C. *sativa*'s biofuel utility. We tested the effect of enzyme concentration and incubation temperature, protoplast purification method, culture media, and culture environment on yield and regeneration. Our objective is to refine the steps of protoplast isolation to enable future work, which may include genetic transformation of C. *sativa* protoplasts and plant regeneration from transgenic protoplasts.

Methods

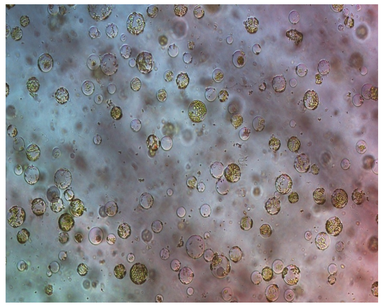


Enzyme Digestion: Three varieties of C. *sativa* seeds were washed with two drops of Tween 20® in tap water for 20 minutes, soaked in 70% ethanol for 5min, rinsed three times with sterile water, soaked with 25% bleach for 10 min, and again rinsed with sterile water until the bleach smell disappeared. Seeds were plated on Murashige and Skoog medium with vitamins (Murashige & Skoog, 1962) containing 3% sucrose, incubated in the dark at 25°C for two days and then moved to light. Two-week-old cotyledons and hypocotyls were placed in 5 ml osmoticum solution in a Petri dish, were finely sliced. The solution was removed after 20 min and incubated for 16 hours in 10mL of enzyme solution containing one of the five combinations of cellulase and macerozyme dissolved in Kao & Michayluk basal salt mixture (Kao & Michayluk, 1975) and 0.4M sucrose or 0.6M mannitol. All digestions were placed in dark on a shaker at 40 rpm and at 25^{o}C. The enzyme combinations are 2% cellulase + 1% macerozyme (C2M1), 1% cellulase + 1% macerozyme (C1M1), 1% cellulase + 0.5% macerozyme (C1M0.5), 0.5% cellulase + 0.25% macerozyme (C0.5M0.25) and 0.25% cellulase + 0.05% macerozyme (C0.25M0.05).

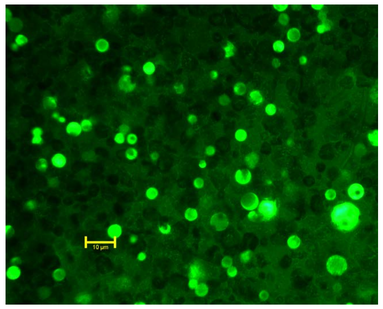
Protoplast Isolation: Digested leaves were gently pipetted . The enzyme solution was filtered through a 60um sieve into a 15mL tube. Protoplast isolation was performed by using two floating and one non-floating method. The first floating method was to layer 1ml PNT (Kao medium, 0.5mg/L NAA, 1.0mg/L BAP, 0.4M Glucose) on the enzyme solution, centrifuge at 100g for 5 minutes, collect and place the floating protoplast band into a new tube, and add 10ml of PNT followed by centrifuging at 100g for 5 minutes for further washing. The second floating method was to centrifuge the 10mL enzyme solution at 100g for 5 minutes, re-suspend the pellet in 10 ml of 20% sucrose, layer 1mL of 10% mannitol, and centrifuge at 200 g for 10 minutes. The protoplast band, appearing in the interface, was collected and placed in a new tube and washed with 10ml PNT. The non floating method was pelleting the protoplasts by centrifuging at 100g for 5 minutes, re-suspending the pellet in 10 ml of a washing solution (0.154 M NaCl, 0.125 M CaCl2-2H2O, 0.005 M KCl, 0.005 M glucose) and centrifuging at 100g for 5 minutes. The final pellets of all the isolation methods were re-suspended in 1mL of KM8 (0.1mg/l 2,4-D, 0.2mg/l Zeatin, 1.0mg/l NAA, 20g/l sucrose, 10g/l Glucose, Kao medium).

Protoplast Assessment and Culture: Protoplast yield (protoplasts/gram fresh tissue) was assessed using disposable hemocytometers (INCYTO C-Chip). FDA stain, prepared by adding 1 drop of a 5mg FDA dissolved 1ml acetone solution into 10mL PNT (working solution), was used to calculate percent viability. One drop of working FDA stain was mixed with one drop of re-suspended protoplasts and observed under a fluorescence microscope after 5 min. KM8 was used to dilute protoplasts to a density of 2x 10^5/ ml . 2ml of 2x 10^5/ ml density protoplasts were diluted with 2 ml liquid KM8 medium (liquid culture). Remaining protoplasts were mixed in a 1:1 ratio with KM8 medium w/ 1.2% agarose solution, again, for a final plating density of 10^5/ ml. Solidified medium was cut into six slices. The slices were transferred to new plates, each containing two slices , thus resulting in three replica plates. In two of the plates, 3 ml of liquid KM8 was added. One solid plate overlayed with liquid KM8 was incubated in 25^{o}C in dark , all other plates of each treatment were incubated in light at 25^{o}C.

Results



C2M1 before filtering (7/13/12)



C1M1 FDA stain (8/1/12)

Enzyme Digestion Temperature

	25^{o}C	28^{o}C
Yield (protoplasts/g fresh weight)	4.45x10^6	1.25x10^6
Percent Viability	38.63	0.00
Viable Protoplast Yield (protoplasts/g fresh weight)	1.72x10^6	0.00

Enzyme Concentration

	C2M1	C1M1	C1M0.5	C0.5M0.25	C0.25M0.05
Yield (protoplasts/g fresh weight)	5.5x10^6	5.65x10^6	4.05x10^6	4.3x10^6	4.1x10^6
Percent Viablity	5.42	38.28	6.73	24.54	24.56
Viable Protoplast Yield (protoplasts/g fresh weight)	2.98x10^5	2.16x10^6	2.72x10^5	1.06x10^6	1.01x10^6

Protoplast Purification Methods

	Sucrose+PNT	Mannitol + Floating on Sucrose	Mannitol+Washing Solution
Yield (protoplasts/g fresh weight)	4.00x10^5	1.00x10^6	5.2x10^6
Percent Viability	4.82	3.23	39.90
Viable Protoplast Yield (protoplasts/g fresh weight)	1.93x10^4	3.23x10^4	2.07x10^6

*Above values are based upon averages.

Conclusions

- Compared to incubation at 25^{o}C, enzyme incubation at 28^{o}C produced 1/4th the number of protoplasts and protoplasts were not viable.
- All five enzyme treatments tested produced similar numbers of protoplasts. However, protoplast viability of C1M1 treatment was considerably higher and that of C2M1 and C1M0.5 was lower.
- Among the three purification methods tested, simple washing of pellet with washing medium yielded significantly higher number of viable protoplasts.
- No data from cultured protoplasts is yet available to report due to their short period of time in culture.

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