Title

Protoplast Regeneration and Its Use in New Plant Breeding Technologies

Authors

Kelsey M. Reed and Bastiaan O. R. Bargmann

Affiliation

School of Plant and Environmental Sciences, College of Agriculture and Life Sciences, Virginia Tech, Blacksburg, VA

Abstract

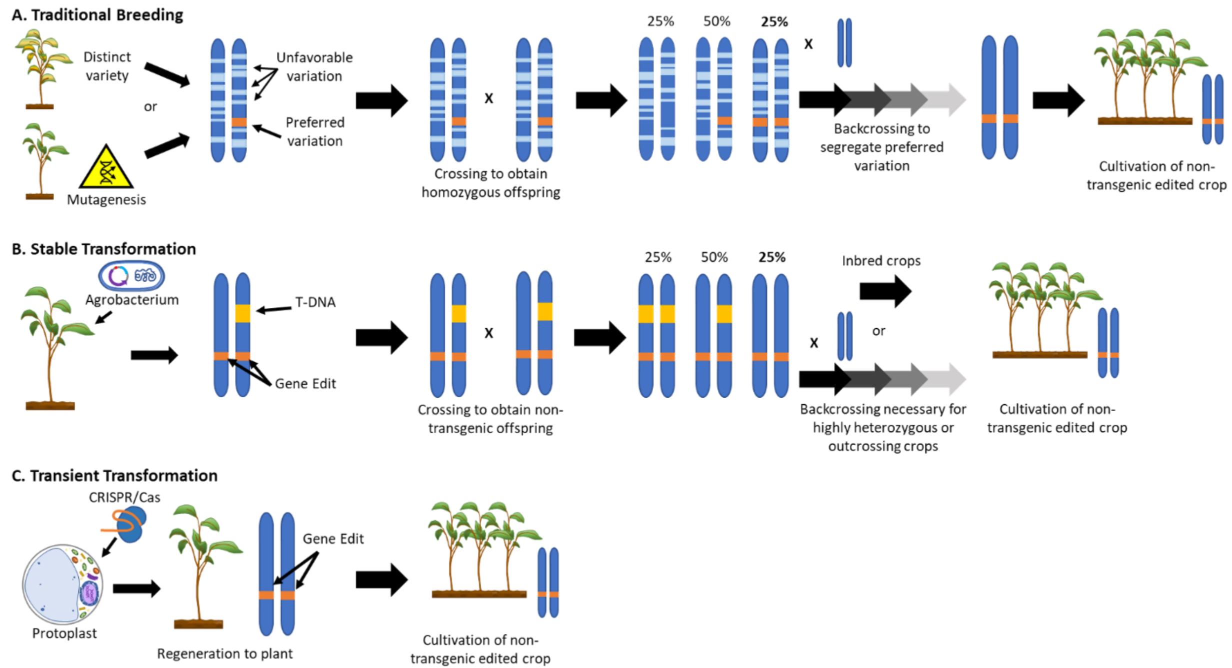
The development of gene-editing technology holds tremendous potential for accelerating crop trait improvement to help us address the need to feed a growing global population. However, the delivery and access of gene-editing tools to the host genome and subsequent recovery of successfully edited plants form significant bottlenecks in the application of new plant breeding technologies. Moreover, the methods most suited to achieve a desired outcome vary substantially, depending on species' genotype and the targeted genetic changes. Hence, it is of importance to develop and improve multiple strategies for delivery and regeneration in order to be able to approach each application from various angles. The use of transient transformation and regeneration of plant protoplasts is one such strategy that carries unique advantages and challenges. Here, we will discuss the use of protoplast regeneration in the application of new plant breeding technologies and review pertinent literature on successful protoplast regeneration.

1. Introduction

Since the advent of CRISPR/Cas9 and related gene-editing technology, direct modification of crop genomes has become the way of the future for advanced breeding techniques in agriculture (Zhang et al., 2019). These new plant breeding technologies (NPBT) have opened avenues of fundamental and translational research that were previously inaccessible. In contrast to transgenic approaches, NPBT can avoid costly and time-consuming regulatory hurdles and accelerate the introduction of new crop lines to the ag market (Lassoued et al., 2021).

Breeding for the introgression of new traits from a wild relative or mutagenized population into an elite crop cultivar is a lengthy procedure, requiring numerous rounds of selection to regain the characteristics of the parental strain (Figure 1A). The ability to efficiently modify crop genes can save several years over conventional breeding approaches and phenotypic recurrent selection (Bull et al., 2017). However, the current most commonly used NPBT method of inserting a transgenic CRISPR/Cas9 construct into the host genome and then crossing it out again to obtain transgene-free progeny still requires multiple rounds of selection (Figure 1B). This is especially true for highly heterozygous and/or outcrossing crops.

In contrast to conventional breeding or transgenic CRISPR/Cas9 approaches, gene editing through transient transformation and regeneration of protoplasts can achieve the desired genetic outcome within a single clonal generation by avoiding the integration of foreign DNA into the host genome (Figure 1C). Aside from the potential to speed up the application of NPBT, the use of protoplasts may have numerous other advantages.



51
52 **Figure 1. Generational Timelines of Traditional and New Plant Breeding Technologies.**
53
54 ## 1.1 Advantages of using protoplasts in NPBT
55
56 As stated above, the use of transient transformation of protoplasts can circumvent transgenesis
57 (the integration of genetic material from one organism into the genome of another organism). The
58 enzymatic removal of the plant cell wall allows for the introduction of foreign DNA, RNA, or protein
59 into protoplasts through either polyethylene glycol (PEG) treatment or electroporation. Although
60 relatively infrequent, the use of DNA (often in the form of plasmids) does not fully preclude the
61 random integration of transgenes (Lin et al., 2018). However, CRISPR/Cas9 can also be
62 expressed through transformation with mRNA encoding the Cas9 enzyme along with the desired
63 guide RNA (gRNA) (Zhang et al., 2016). Alternatively, protoplasts can be transformed with
64 ribonucleoprotein complexes, consisting of Cas9 associated with the gRNA (Svitashev et al.,
65 2016). The latter two approaches more effectively preclude the integration of foreign DNA,
66 although there have been cases where DNA-template contamination in the *in vitro* transcribed
67 mRNA or gRNA has led to insertions, *e.g.* (Andersson et al., 2018). Particle bombardment is a
68 potential alternative for transient delivery method for DNA-free gene-editing tools, *e.g.* (Liang et
69 al., 2018). However, it may suffer from limitations in transformation efficiency and the regeneration
70 of chimeric plants (as discussed below).
71
72 If the goal of the gene-editing approach goes beyond site-specific insertions and/or deletions for
73 the knock-out of gene function but instead aims for specific nucleotide substitutions or insertion
74 of a specific sequence through homologous recombination, there is a need for the co-introduction
75 of a DNA-repair template (as in oligo directed mutagenesis) or a donor sequence, respectively.
76 Prime editing and viral replicons are potential methods to deliver such templates and donors
77 transgenically (Čermák et al., 2015; Lin et al., 2020). However, in addition to the potential for a
78 non-transgenic outcome, the use of protoplasts allows for more control over the amount of
79 template or donor delivered and effect higher precision and efficiency, *e.g.* (Sauer et al., 2016).
80

In many plant species, the lack of host susceptibility to *Agrobacterium* transformation limits the use of transgenic NPBT approaches. This is seen in particular in monocots (Hwang et al., 2017). Host-pathogen incompatibility is also expected to be a limiting factor in the applicability of viruses for the delivery of gene-editing tools (Ma et al., 2020). In such cases, the use of protoplasts (or particle bombardment) may be a feasible alternative delivery method.

Chimerism (where only parts of the regenerated plant are descended from an edited cell) can be an issue when using conventional, tissue-culture based approaches where a callus intermediate is used, *e.g.* (Charrier et al., 2019). This phenomenon occurs because *de novo* shoots or embryos can be formed from a group of cells rather than a single antecedent. In the case of protoplasts, regenerated plants are (in most cases) derived from a single cell, thereby avoiding this potential problem. Chimerism can be a concern especially when non-selectable, non-transgenic approaches are used together with conventional tissue culture, *e.g.* transient transformation with Agrobacterium or particle bombardment. Additionally, such non-selectable strategies can suffer from low editing efficiency in the regenerated plants because only the cells on the surface of the tissue are potentially edited whereas regeneration can also occur from the numerous non-transformed cells. In comparison, protoplast transformation efficiencies are much higher and plants regenerated from protoplasts transiently transformed with editing tools will therefore have better chance of being successfully edited.

However, a glaring limitation in the use of protoplasts for NPBT is the challenges faced in the regeneration of plants from single cells and there appears to be no universal strategy that applies to diverse (sub)species. Plant tissue culture in general, and protoplast regeneration in particular, is often lightheartedly considered more of an artform than a science, requiring an experienced eye and instinctual decision making, as comprehensive systematic approaches are too vast in scope to be feasible. In this review, we will discuss a compilation of literature on plant regeneration from protoplasts. We will deliberate protoplast isolation, protoplast culture, and plant regeneration from protoplast culture, specifically in the light of the application of NPBT.

2. Obtaining Protoplasts

2.1. Source Tissue

The tissue from which protoplasts are derived is very important for obtaining regenerable starting material. The genotype, organ or tissue, and growth conditions of the plants used can be a significant determinant in regeneration success.

2.1.1. Genotype

Different cultivars or ecotypes can have widely varying success rates in tissue culture and protoplast regenerative capacity. Depending on the species being worked with and the end goal of the application, it is recommended to assess the regenerative capacity of multiple genotypes and select the most suitable for further use.

When comparing four different Arabidopsis (*Arabidopsis thaliana*) ecotypes (Col-0, Ws-2, No-0, and HR-10), all gave a similar number of protoplasts with an optimized digestion, but differed significantly when comparing optimal protoplast division media, callus induction media, and shoot induction media (Jeong et al., 2021). Ws-2 showed the highest regeneration efficiency, whereas

the Col-0, No-0, and HR-10 had relatively ineffective regeneration rates, regardless of efforts to vary the composition of media and tissue culture methods.

Comparison of three different *Cyclamen* species (*C. graecum*, *C. mirabile*, and *C. alpinum*) found significant differences in protoplast culture and regeneration, including division frequencies (often referred to as plating efficiency) and morphological appearance of regenerating embryos (Prange et al., 2010a). Plants were regenerated from protoplasts derived from embryogenic callus in all three species, but had different efficiencies in microcallus formation and development of somatic embryos. Interestingly, there was no correlation between the regenerative capacity of the source embryogenic callus and the ability of the protoplasts to divide and regenerate, with *C. graecum* performing the worst in regeneration from callus but showing the highest protoplast division rates.

2.1.2. Organ or Tissue

Different source materials for protoplast isolation can affect the number, size, viability, and regenerative capacity of protoplasts. There are examples of protoplast isolation and regeneration from numerous tissues, including leaves, cotyledons, roots, petioles, hypocotyls, petals, callus, and suspension cultures (Table 1).

In cabbage (*Brassica oleracea*), it was observed that hypocotyl-derived protoplasts yielded more regenerated shoots than leaf-derived protoplasts (Kiełkowska and Adamus, 2012). In a comparison on the regeneration capacity of protoplasts derived from leaves, cotyledons, and callus from coastal medick (*Medicago littoralis*), leaf protoplast-derived callus was found to have the highest regeneration capacity with a frequency of 20% and cotyledon protoplast-derived callus had a regeneration frequency of 15% (Zafar et al., 1995). In this study, callus-derived protoplasts developed only a few microcolonies that were not tested for regeneration. Embryogenic callus can potentially provide improved regeneration success in cases where somatic tissues fail to produce regenerable protoplasts, *e.g.* in grapevine (*Vitis vinifera*) (Bertini et al., 2019).

The age of the source tissue can also be of importance, both for protoplast yield and viability as well as regeneration success. Generally, protoplasts derived from younger tissues perform better in culture. This has been shown for hypocotyls and leaves in cabbage (Kiełkowska and Adamus, 2012) and cell suspension culture in oil palm (*Elaeis guineensis*) (Masani et al., 2013), for example.

2.1.3. Plant Growth Conditions

The growth conditions of the starting material, including growth media and light, can have a significant effect on the regenerative capacity of protoplasts. An important consideration is that the material needs to be sterile (either grown under aseptic conditions or sterilized upon harvest) in order to be used for further culture of the obtained protoplasts.

In Arabidopsis, plants grown on Gamborg B5 medium and harvested three weeks after germination had a larger rosette with nearly twice as many leaves when compared to plant grown on Murashige and Skoog (MS) medium, resulting in twice as many protoplasts per harvested plant. However, during protoplast culture, the plants grown initially on MS media showed two to three times higher plating efficiency. And when comparing the photoperiod under which plants were grown, short day (10 h) resulted in a fourfold higher plating efficiency than long day (16 h) (Masson and Paszkowski, 1992).

Examination of cauliflower (*Brassica oleracea*) leaf protoplast quality of shoots grown in various vessel types found that protoplast yield, viability, division, and shoot regeneration was higher from tissue of plants grown in containers with vented lids compared to containers with closed lids (Chikkala et al., 2009).

2.2. Enzymolysis

When it comes to isolating protoplasts, it is not only about obtaining a high number of protoplasts, but also about optimizing their viability and regenerative capacity. Many factors in the enzymolysis procedure may be of influence, including the utilized pretreatment, buffer composition, cell-wall digestion enzymes, incubation conditions, and purification methods (Table 1). Although, to our knowledge, there are not studies on the effect on protoplast regeneration directly for all of the different factors described here, it seems reasonable to assume that effects on the quality (viability) of the isolated protoplasts will translate to an influence on regenerative capacity of the isolated protoplasts.

2.2.1. Pretreatment

Pretreatment of tissue can be used to augment the number of viable protoplasts isolated by increasing the access of the used enzymes to the plant cell wall. This can be achieved through physical disruption of the tissue (*e.g.* slicing leaf tissue), vacuum infiltration of the enzyme solution, or a preplasmolysis treatment.

Slicing tissue into smaller sections or strips before moving to the enzyme solution allows for more surface area for the enzymes to work, leading to the release of more protoplasts. With rice (*Oryza sativa*), longitudinal cutting, parallel to the veins, before enzyme digestion resulted in over twice as many viable protoplast as leaves cut in cross section (Lin et al., 2018). Another example of physical disruption is the “Tape-Arabidopsis Sandwich” method (Wu et al., 2009). This method uses tape on both sides of a leaf to add support and allow the removal of the bottom epidermal layer. This protocol has been successfully applied to other Brassicaceae species, including *B. oleracea*, *B. napus*, *Cleome spinosa*, *C. monophilla*, and *C. gynadra* (Lin et al., 2018).

In addition to physical disruption, vacuum infiltration of plant tissue with the enzyme solution can be used to ensure that the enzymes are able to reach more of the cells, which could increase protoplast yield. In both apple (*Malus domestica*) and grapevine, vacuum infiltration was a part of the optimization of the protoplast isolation procedure to obtain the highest number of viable protoplasts per gram of fresh weight (Osakabe et al., 2018).

Preplasmolysis treatment is used to shrink the protoplasts away from the cell wall before introducing the enzyme solution. This is thought to avoid damage to the cell membrane. When comparing protoplasts isolated from birdsfoot trefoil (*Lotus corniculatus*) tissue with and without preplasmolysis, the pretreated protoplasts had roughly five times more cell wall formation than the nontreated after 3 days of culture. After one week, the viability of the nontreated protoplasts decreased significantly (Vessabutr and Grant, 1995).

230 *2.2.2. Enzyme Solution Buffer*
231
232 The buffer for the enzyme solution is critical for optimal enzyme activity and ensuring a high
233 number of viable protoplasts. The buffer solution typically includes KCl; $CaCl_2$; mannitol, sorbitol,
234 or salts as osmolytes; MES (2-(N-morpholino)ethanesulfonic acid) as pH buffer; BSA as an
235 alternate target for proteases that may degrade the enzymes; and β-mercaptoethanol as a
236 reducing agent (Table 1). Frearson et al. (1973) first formulated a combination of salts that many
237 still use, called the cell and protoplast washing (CPW) salts. This basal salt solution is often
238 modified with the addition of mannitol or sorbitol for osmotic pressure and different enzymes for
239 optimal protoplast isolation (Tomiczak et al., 2015; Jones et al., 2015; Jie et al., 2011).
240
241 Proper osmolality is crucial in order to ensure the survival of the cells and provide an environment
242 for potential cell wall formation and division, leading to regeneration. Protoplast development has
243 been shown to be inhibited by excess osmotic pressure during isolation and culture by impairing
244 metabolism (Ruesink, 1978) as well as division and cell wall regeneration (Pearce and Cocking,
245 1973).
246
247 Enzyme solutions with the same (or similar) composition as the subsequent protoplast culture
248 medium have also been used successfully in protoplast regeneration applications. For example
249 sugar beet (*Beta vulgaris*) callus protoplasts were isolated using Kao and Michayluk salts in the
250 enzyme solution (Dovzhenko and Koop, 2003); Mango (*Mangifera indica*) pro-embryogenic mass-
251 derived protoplasts were isolated using an enzyme solution containing Gamborg B5 and
252 Murashige and Skoog salts (Ara et al., 2000); petunia (*Petunia* spp.) and calibrachoa (*Calibrachoa*
253 spp.) leaf protoplasts were isolated with Kao and Michayluk and Gamborg B5 salts in the solution
254 (Meyer et al., 2009).
255
256 *2.2.3. Enzymes*
257
258 Many commercially available cell-wall degrading enzymes (or enzyme mixtures) are used for the
259 isolation of protoplasts. They differ in their substrates as well as the purity or combination of the
260 enzymes in the extract. Enzymolysis is generally achieved using both cellulases and
261 hemicellulases (*e.g.* beta-glucanases, xylanases, protopectinases, polygalacturonases, pectin
262 lyases, and pectinesterases). Some of the most commonly used enzymes or enzyme mixtures
263 are Cellulase R-10, Macerozyme R-10, and Pectolyase Y-23 (Table 1). The manufacturer/supplier
264 of the enzymes may be a factor in the success rates (personal experience and communication
265 with others).
266
267 The effect of different enzyme combinations and concentrations were tested on the isolation of
268 protoplasts from stevia (*Stevia rebaudiana*) leaves (Lopez-Arellano et al., 2014). The optimized
269 enzyme solution contained 2% Cellulase R-10, 1.5% Macerozyme Onozuka R-10, 0.2%
270 Driselase, and 0.1% Pectolyase Y-23. When the Cellulase R-10 was decreased to 1% or
271 increased to 3%, there was a significant drop in both the yield and viability of the protoplasts.
272 There was also a lower viability when Pectolyase Y-23 was not present. When isolating
273 protoplasts from tobacco (*Nicotiana tabacum*) leaves, it was found that Pectolyase Y-23 was 20
274 times more effective than Macerozyme R-10 (Nagata and Ishii, 1979). This was determined to be
275 due to the Pectolyase Y-23 having 50 times stronger endopolygalacturonase activity.
276
277 As the cost of lab-grade enzymes can be prohibitive, the use of food-grade cell wall degrading
278 enzymes was investigated as a low-cost alternative for the isolation of switchgrass (*Panicum*
279 *virgatum*) leaf protoplasts (Burris et al., 2016). It was determined that using a combination of

Rohament CL with Rohapect 10L and Rohapect UF (cellulases and pectinases commonly used in brewing and juicing) yielded up to 8.4 x 10^5 protoplasts per gram of leaf tissue.

Although (to our knowledge) there have been no systematic analyses of whether the combination of enzymes used may influence the division rates and regenerative capacity of the produced protoplasts, one can imagine that there could well be an effect. The enzymes themselves, the crude extracts, as well as the cell-wall degradation products they produce can all be recognized by plant cells as pathogenic elicitors, to a greater or lesser extent, depending on the sensitivity of the genotype used to the different enzymes and extracts employed. Protoplast yield and viability may well be a good measure for protoplast isolation, but it could be the case that an enzyme combination that does not necessarily give the highest yield and viability could be more suitable for subsequent regeneration of the protoplasts.

2.2.4. Enzymolysis Conditions

Conditions during protoplast isolation (*i.e.* duration, temperature, light, and agitation) can play a significant role in the subsequent yield, viability and regenerative capacity of the protoplasts.

The length of a digestion period typically ranges from 2 to 18 hours (Table 1). The duration of digestion needs to be long enough to release sufficient numbers of protoplasts, but not too long as to decrease the viability due to cell damage or the lack of nutrients and growth regulators in the enzymolysis solution. For example, when comparing 4, 8, and 12 h digestion duration of crown imperial (*Fritillaria imperialis*) callus, the yield and viability were highest at 8 h (Chamani and Tahami, 2016).

Temperature also plays an important role in protoplast yield and viability. Room temperature is the most commonly used, although there are examples of higher temperatures being employed (Table 1). There could be effects on enzyme activity (and protoplast yield) as well as protoplast viability and regenerative capacity. Intuitively, it may be preferable to use a temperature that is close to that used for the growth of the source material and/or subsequent protoplast culture conditions, in order to minimize temperature fluctuations or shocks. Conversely, perhaps a particular temperature treatment may actually benefit regenerative capacity.

Digestion in a light or dark condition may additionally influence the protoplast isolation, with most choosing dark conditions (Table 1). This may avoid the production of free radicals and photoinhibition in cells containing chloroplasts. Although there are also examples where digestion under light performed better than in the dark. In geranium (*Pelargonium* x *hortorum*) leaf protoplast isolation, protoplast yield and viability were increased when the digestion occurred in light; in the dark, the enzymes were efficient but most of the released protoplasts had burst (Nassour and Dorion, 2002). The protoplasts isolated from the light condition were regenerated into plants, but the effect of light or dark condition during digestion on the regeneration capacity was not investigated.

Agitation of the enzymatic solution on a gyratory shaker during the protoplast digestion can increase the protoplast yields. Typically, speeds range from 0 to 90 rpm, with the average being around 40 rpm (Table 1). Alternatively, the agitation can be implemented only at the end of the digestion period to facilitate the release of protoplasts from the cell wall remnants.

329 Again, protoplast yield and viability may well be a good measure, but it could be the case that
330 digestion conditions that do not necessarily give the highest yield and viability could be more
331 suitable for subsequent regeneration of the protoplasts.
332
333 *2.2.5. Purification*
334
335 Following enzymolysis, separation of the protoplasts from undigested tissue, cell wall debris, and
336 dead cells can be an important factor in the culture of the protoplasts. Debris and dead cells may
337 elicit negative effects in the living protoplasts that will inhibit their division and development, *e.g.*
338 in kalanchoe (*Kalanchoe blossfeldiana*) (Castelblanque et al., 2010). Filtration and sucrose
339 cushions, or floatation through a density gradient, are commonly used techniques.
340
341

342 **3. Protoplast Culture**
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344 <u>3.1. Culture Media</u>
345
346 Protoplast culture media are central to protoplast division and plant regeneration. The appropriate
347 macro-, micro-nutrients, and additives, such as plant growth regulators, osmotic stabilizers,
348 medium solidifiers, and supplements, are essential in protoplast culture.
349
350
351 *3.1.1. Nutrients*
352
353 Optimal protoplast culture media vary widely, depending on the genotype and source tissue used
354 (Table 2). Common medium formulations (such as MS (Murashige and Skoog, 1962), Gamborg
355 (B5) (Gamborg et al., 1968), Kao and Michayluk (KM (Kao and Michayluk, 1975)), Y3 (Eeuwens,
356 1976), or Nitsch (Nitsch and Nitsch, 1969)), or slight modification thereof, are often used in
357 protoplast culture. Although there are also examples of custom formulations, *e.g.* TM2G for
358 tomato (*Solanum lycopersicon*) protoplast culture (Shahin, 1985). This is also a case where the
359 manufacturer/supplier of the premixed media may be a factor in the success rates (personal
360 experience and communication with others). When establishing and optimizing a protoplast
361 culture procedure, it is prudent to assay an array of medium formulations for suitability.
362
363 In a comparison of 14 formulations based on MS, KM, and Y3 media for oil palm cell suspension-
364 derived protoplast division, Y3-based medium gave the fastest cell wall formation, quickest
365 division, and highest division frequency (Masani et al., 2013). Amur cork tree (*Phellodendron*
366 *amurense*) stem protoplasts were cultured in MS, half-strength MS, and Woody Plant Medium
367 (WPM), and culture in full-strength MS medium resulted in the highest colony formation rate
368 (Azad, 2012).
369
370 Protoplast cultures also need a carbon source for energy metabolism, typically sucrose or glucose
371 and to a lesser degree mannitol or sorbitol (Table 2). Comparing the effect of 1% and 2% of either
372 glucose or sucrose as the carbon source for chrysanthemum (*Chrysanthemum morifolium*) leaf
373 protoplast culture, 1% sucrose performed best (Adedeji et al., 2020). Although 2% sucrose
374 resulted in the highest division rate, there was no subsequent colony formation. Only 1% sucrose
375 and 2% glucose led to microcallus formation, with 1% sucrose more rapidly producing larger
376 microcalli. For Arabidopsis seedling protoplast culture, three different variations of supplements
377 with B5 medium and vitamins were tested for protoplast proliferation (Jeong et al., 2021). Myo-

378 inositol as the primary carbon source along with sucrose resulted in the highest proliferation rate
379 across four the different Arabidopsis ecotypes. A simplification of KM8p medium with the removal
380 of all of the sugars (fructose, ribose, xylose, mannose, rhamnose, cellobiose, sorbitol and
381 mannitol) except glucose still resulted in protoplast division that led to callus and embryo formation
382 from carrot (*Daucus carota*) leaf protoplasts (Grzebelus et al., 2012).
383
384

385 *3.1.2. Osmotic Pressure*

386
387 Osmotic pressure is an important aspect of protoplast culture media. Generally, mannitol, sorbitol,
388 sucrose, glucose, myo-inositol or a combination of these components is used to ensure the proper
389 osmolarity. Determining the proper solute concentration is critical for the protoplast survival and
390 division rates. Generally, the concentration of the major osmoticum used in the initial protoplast
391 culture medium varies from 0.1 to 0.8 M (Table 2). Intuitively, it seems that having a comparable
392 osmolarity between enzymolysis and initial culture conditions would expose the protoplasts to
393 less osmotic shock upon transfer to culture medium and benefit their viability and vigor.
394
395 For cabbage cotyledon protoplasts, myo-inositol was a better osmotic regulator than mannitol (Jie
396 et al., 2011). It is theorized that myo-inositol may be advantageous to both carbohydrate
397 metabolism in cell walls and inositol metabolism in cell membranes in protoplast culture. However,
398 whether these advantages are gained with a small addition of myo-inositol with a different primary
399 osmoticum or if a large quantity of myo-inositol is needed has yet to be determined.
400
401 Osmolarity is commonly decreased gradually as the protoplast reform their cell walls and begin
402 to divide. For example, gradually reducing the osmolarity for oil palm cell suspension protoplast
403 cultures doubled the number of microcalli (Masani et al., 2013). In gentian (*Gentiana decumbens*)
404 leaf protoplast culture, the osmolarity of the liquid medium around agarose beads was decreased
405 by reducing the mannitol concentration from 0.5 M to 0.33 M during the fifth and sixth week of
406 culture, followed by another decrease to 0.17 M mannitol in the seventh and eighth week, and no
407 mannitol for the subsequent weeks (Tomiczak et al., 2015). In chrysanthemum protoplast culture,
408 after the first week in liquid culture medium, myo-inositol was omitted from the refresh medium
409 and mannitol concentrations were dropped from the initial 0.4 M to 0.32, 0.21, and 0.11 M for
410 weeks 2, 3, and 4, respectively (Eeckhaut et al., 2020).
411
412

413 *3.1.3. Plant Growth Regulators*

414
415 Plant growth regulators, particularly cytokinins and auxins, are essential for the growth of
416 microcalli from protoplasts. Additionally, gibberellic acid (GA_3) has been shown to be beneficial in
417 some cases. The most common cytokinins are 6-benzylaminopurine (BAP), zeatin, kinetin,
418 isopentenyl adenine (2iP), and thidiazuron (TDZ). The most common auxins are indole-3-acetic
419 acid (IAA), indole-3-butyric acid (IBA), 2,4-dichlorophenoxyacetic acid (2,4-D), and naphthalene
420 acetic acid (NAA). Optimal concentrations, combinations, and ratios vary widely, depending on
421 the genotype and source tissue of the protoplasts (Table 2).
422
423 A ratio of a relatively higher concentration of auxin with a lower concentration of cytokinins was
424 effective for microcallus formation from populus (*Populus beijingensis*) cell suspension
425 protoplasts (Cai and Kang, 2014). Conversely, in kalanchoe leaf protoplast culture, a higher
426 cytokinin to auxin ratio resulted in better proliferation and microcallus formation; having cytokinin
427 exclusively resulted in slow growth and the microcalli eventually died (Castelblanque et al., 2010).

428
429 Coconut water is a natural source of plant growth regulators, both auxin (IAA) and cytokinins
430 (various) as well as other phytohormones, such as gibberellins, and other supplements, such as
431 vitamins and minerals, that have been found to be beneficial in plant tissue culture (Yong et al.,
432 2009). As a supplement in corn (*Zea mays*) embryogenic callus protoplast culture, coconut water
433 led to a high efficiency of microcallus formation, with a 2% coconut water addition producing the
434 most microcalli (Imbrie-Milligan et al., 1987). Coconut water was also found to increase protoplast
435 cell division in orchid (*Phalaenopsis* spp.) callus protoplasts (Kobayashi et al., 1993).
436
437
438 *3.1.4. Additional Supplements*
439
440 Additional supplements, such as polyvinylpyrrolidone, antioxidants, activated charcoal, silver
441 nitrate, antibiotics, complex organics, amino acids, polyamines, conditioned medium, and peptide
442 growth factors, can be added to the media to support protoplast division and microcallus formation
443 (Table 2).
444
445 Antioxidants, such as ascorbic acid, citric acid, reduced glutathione, and L-cysteine, can be used
446 to mitigate the inhibitory effects of reactive oxygen species. In oil palm protoplast regeneration, it
447 was found that 200 mg/L ascorbic acid gave the greatest indication of further cell growth and
448 development with the microcalli turning yellow and developing into embryogenic calli (Masani et
449 al., 2013). With this supplementation, two types of embryogenic callus were observed, compact
450 and friable embryogenic callus, which were both able to further develop into somatic embryos and
451 regenerate into plantlets.
452
453 Polyvinylpyrrolidone (PVP) is used to adsorb phenolics. While phenolics may be beneficial for
454 plant defense (Bhattacharya et al., 2010), an accumulation during protoplast culture has been
455 found to lead to oxidative browning of the culture medium, inhibiting protoplast growth and division
456 (Reustle and Natter, 1994; Prakash et al., 1997). There has also been reports of PVP suppressing
457 tissue browning and improving callus formation in peony (*Paeonia lactiflora*) petal explant tissue
458 culture (Cai et al., 2020). Polyvinylpolypyrrolidone (PVPP), a highly cross-linked version of PVP,
459 has also been found to inhibit tissue necrosis in Virginia pine (*Pinus virginiana*) callus culture
460 (Tang et al., 2004), as well as preventing browning better than PVP in guar (*Cyamopsis*
461 *tetragonoloba*) cotyledon protoplast culture (Saxena and Gill, 1986). When PVP was added to the
462 PVPP culture of guar cotyledon protoplasts, not only was it found to enhance the necrosis
463 inhibition, but it also improved the protoplast division frequency. Another compound known to
464 decrease tissue browning is 2-aminoindane-2-phosphonic acid (AIP), which is a reversible
465 inhibitor of phenylalanine ammonia lyase (PAL), an enzyme necessary for polyphenol production
466 (Appert et al., 2003). While the inhibition of PAL was able to increase the cell wall digestibility and
467 facilitate sustained cell division in American elm (*Ulmus americana*), extended inhibition results
468 in decreased shoot growth in tissue culture (Jones et al., 2012). This decrease in plant growth
469 due to PAL inhibition from AIP has also been seen in birch (*Betula pubescens*) (Nybakken et al.,
470 2007) and St. John's wort (*Hypericum* spp.) (Klejdus et al., 2013). It could be hypothesized that
471 an early addition of AIP will increase the likelihood of protoplast survival, but it should not be used
472 for an extended period as to disrupt the callus and shoot growth, as described for American elm
473 protoplast regeneration (Jones et al., 2015).
474
475 Activated charcoal is a commonly used additive employed for its ability to adsorb inhibitory
476 elements, such as phenolics and reactive oxygen species, that can impede protoplast division.
477 Adedeji et al. (2020) found that the ideal concentration of activated charcoal for chrysanthemum
478 leaf protoplast regeneration was 0.02% (w/v) and adding a higher concentration of 0.1% resulted

479 in agglutination of the protoplasts, causing them to die before entering the microcolony stage. In
480 primrose (*Primula* spp.) cell suspension-derived protoplast culture, the addition of 0.1% PVP did
481 not induce callus formation; however, the addition of activated charcoal did (Mizuhiro et al., 2001).
482
483 Silver nitrate ($AgNO_3$), an inhibitor of ethylene action, has been shown in some cases to increase
484 callus formation and regeneration efficiency as well as effect protoplast isolation efficiency. The
485 culture of hypocotyl protoplasts from several *Brassica* species was markedly improved by the
486 addition of silver nitrate in the culture medium (Pauk et al., 1991; Hu et al., 1999). With rice (*Oryza*
487 *sativa*) suspension cultures, the addition of silver nitrate during protoplast isolation reduced
488 protoplast yield but increased the frequency of colony formation (Ishii, 1988).
489
490 Antibiotics may be used to avoid endogenous or exogenous contamination, however they can
491 either inhibit or stimulate explant growth and development with the direct causation not yet
492 understood (Qin et al., 2011). A study analyzing the effects of three β-lactam antibiotics
493 (cefotaxime, carbenicillin, and timentin) at different concentrations on carrot seedling protoplasts
494 found that, while plating efficiencies decreased in all antibiotic concentrations higher than 100
495 mg/L, cefotaxime and timentin in the range of 100-500 mg/L increased regeneration efficiency
496 (Grzebelus and Skop, 2014). Timentin was used with *Hydrangea* leaf protoplasts to limit the
497 endophytes and it was observed that in antibiotic-free medium, the protoplasts rebuilt the cell wall
498 faster and divided earlier, but callus was only formed in medium with antibiotics (Kästner et al.,
499 2017).
500
501 The exact composition of complex organics, such as casein hydrolysate, casamino acids, coconut
502 water, and yeast extract, is typically undefined and varies depending on the manufacturer/supplier
503 and potentially the batch. However, the amino acids, hormones, vitamins, fatty acids,
504 carbohydrates, and other growth supplements they provide may enhance growth and
505 regeneration of plants (Bhatia, 2015). The addition of casein hydrolysate was initially shown to
506 give a more consistent high rate of microcallus formation from tobacco (*Nicotiana tabacum*)
507 protoplasts (Galun and Raveh, 1975), and is currently an addition to protoplast culture media
508 regularly (Table 2).
509
510 Polyamines can regulate plant growth and stress responses through many means, including
511 increasing antioxidant activity and regulating oxidative stresses (Chen et al., 2019). In a
512 comparison of the exogenous addition of the polyamines putrescine, spermidine, and spermine
513 on sugar beet (*Beta vulgaris*) cell suspension-derived protoplasts, spermine resulted in the
514 highest plating efficiency, likely due to its stronger inhibitory effect on ethylene production
515 (Majewska-Sawka et al., 1997). Polyamines exogenously applied in different concentrations on
516 cabbage hypocotyl protoplast culture obtained the highest frequency of shoot organogenesis from
517 protoplasts treated with putrescine (Kiełkowska and Adamus, 2021). However, the addition of
518 putrescine had no effect on the culture or regeneration of Love-in-a-Mist (*Nigella damascena*)
519 callus protoplasts (Klimek-Chodacka et al., 2020).
520
521 Conditioned medium (spent liquid medium used for cell-suspension cultures that is filtered and
522 subsequently used as a supplement for protoplast culture) may contain compounds that
523 encourage growth and mitotic activity. Fresh conditioned medium from cell-suspension cultures
524 significantly increased the plating efficiency in chrysanthemum leaf protoplast culture (Zhou et al.,
525 2005).
526
527 Phytosulfokine (PSK), specifically PSK-α, is a peptide that was originally detected secreted in
528 conditioned medium, but was later found in whole plants (Yang et al., 1999). It was found to
529 promote cell growth, enhance callus growth as well as adventitious root and bud formation, and

improve somatic embryogenesis in multiple species, and has also been shown to enhance protoplast regeneration in carrot (Maćkowska et al., 2014) and cabbage (Kiełkowska and Adamus, 2019). With carrot leaf protoplasts, application of PSK-α during the initial culture resulted in a four-fold increase in yield of regenerated plants (Maćkowska et al., 2014). PSK-α was shown to be both genotype- and dose-dependent and did not require a constant presence to maintain cell divisions in cabbage leaf protoplasts (Kiełkowska and Adamus, 2019). Not only was the PSK-α found to promote cell proliferation, but it also increased differentiation and organogenesis in five of the six cabbage accessions tested.

3.2. Protoplast Culture Conditions

Protoplast culture conditions, such as the use of liquid or semi-solid medium, temperature and light, cell density, or the presence of nurse cultures, can have a significant effect on the division and microcallus formation potential of protoplasts.

3.2.1. Liquid vs Semi-solid Medium

When it comes to determining the solidity of the media to use with protoplast culturing, there are multiple factors to consider, including imaging potential, media refreshing, toxin accumulation, and cell aggregation.

Liquid medium is the most straightforward to make since it requires no agar manipulation. However, it faces a multitude of challenges. With imaging, unless each cell is in a separate space, it is impossible to track the growth of an individual cell. There is also the potential for aggregation of cells to form a non-homogeneous callus, possibly resulting in chimerism of the regenerated plants. Aggregation can also cause a local accumulation of toxic substances released from dying cells that may inhibit the growth of neighboring cells (Deryckere et al., 2012).

To avoid cell agglutination, embedding the protoplasts in semi-solid medium can ensure physical separation of cells. The embedding medium will typically contain agar, agarose, or alginate as a solidifier. Alginate is favorable for heat-sensitive protoplasts because the gelling is induced by exposure to calcium ions rather than the need to heat the agar or agarose solutions above the melting point.

In a comparison between thin alginate layers and extra thin alginate films on carrot shoot protoplast culture, thin alginate layers resulted in nearly a 20% increase in plating efficiency in every accession tested (Maćkowska et al., 2014). Sterilizing the alginate solution through filter-sterilization was also found to give over a 10% increase in plating efficiency over autoclave-sterilization in several of the accessions used.

The amount of liquid medium surrounding alginate beads can affect the protoplast proliferation capability. In American elm (*Ulmus americana*) cell suspension-derived protoplast alginate bead culture, cultures that contained less than 2 mL or more than 3 mL of liquid medium failed to develop beyond the first cell division; whereas cultures that contained 2 or 3 mL of liquid medium continued to proliferate (Jones et al., 2015).

579 *3.2.2. Temperature and Light*
580
581 The temperature and light conditions used during protoplast culture vary widely (Table 2) and
582 have both been shown to be of effect in regeneration success. Cabbage leaf protoplast cultures
583 were greatly affected by light and temperature, with very few divisions occurring in cultures moved
584 from dark at 25 °C to light at 23 °C after seven days of culture, compared to those kept in the dark
585 conditions for all 15 days (Kaur et al., 2006). Using lettuce (*Lactuca saligna*) leaf protoplasts, dark
586 culture led to sustained division while light bleached and killed the protoplasts in 3 days (Brown
587 et al., 1987). However, Arabidopsis cotyledon protoplasts did not show a significant variation in in
588 either the plating density or growth rates whether cultured in the light or dark (Dovzhenko et al.,
589 2003).
590
591
592 *3.2.3. Cell Density*
593
594 The protoplast plating density can range from single cells up to a few million protoplasts per
595 milliliter, but typically range from $5x10^4$ – $1x10^6$ protoplast/mL (Table 2). In a comparison of plating
596 densities of petunia (*Petunia hybrida*) leaf protoplast culture, 1 x 10^6 protoplasts/mL produced a
597 significantly higher division frequency and number of calli than 5 x 10^4 protoplasts/mL (Kang et
598 al., 2020). However, the microcolony viability decreased with the plating density increasing to 1.5
599 x 10^6 protoplasts/mL, potentially due to high phenolics accumulation. Over-crowding the
600 protoplasts can also result in a lower viability due to a lack of available nutrients (Kiełkowska and
601 Adamus, 2012). In contrast, a lower density may also be desired to track an individual protoplast
602 after transformation or fusion (Bhojwani and Dantu, 2013). However, a lower protoplast density
603 can be more costly and time consuming. Additionally, protoplasts can release growth factors
604 which can stimulate mitotic division non-cell-autonomously. This is also the basis for nurse
605 cultures.
606
607
608 *3.2.4. Nurse Cultures*
609
610 Nurse cultures are the culture of target protoplasts with additional actively dividing protoplasts or
611 suspension cells, either from the same species (*e.g.* in crocus (*Crocus cancellatus*) embryogenic
612 calli-derived protoplast culture (Karamian and Ebrahimzadeh, 2001)) or from another, often
613 closely related species (*e.g.* in desert banana (*Musa paradisiacal*) embryonic cell suspension
614 protoplast culture (Dai et al., 2010) and cauliflower (*Brassica oleracea* var. *botrytis*) hypocotyl
615 protoplast culture (Sheng et al., 2011)). There are many nurse culture techniques, one example
616 is feeder layer-cultures, which can be embedding the target protoplasts in agar layers with the
617 nurse cells in a liquid surrounding the layers (Sheng et al., 2011), or the target protoplasts in liquid
618 culture with the nurse cells embedded in agarose (Dai et al., 2010). Alginate bead cultures, which
619 can be performed by embedding the target protoplasts in alginate beads and having the nurse
620 cells in liquid medium (*e.g.* with rice (*Oryza sativa*) suspension culture protoplasts (Kyozuka et
621 al., 1987)). An alternate method for ensuring a separation of the nurse cells and the target
622 protoplasts is using a nitrocellulose filter which allows growth factors, signaling molecules, and
623 nutrients to pass through, but not cells (Dai et al., 2010).
624
625
626
627 **4. Plant Regeneration from Protoplast Culture**
628

4.1. Callus Formation

From microcalli, regeneration could come from organogenesis or embryogenesis. Organogenesis-oriented microcalli can be moved to a callus proliferation medium to increase the callus size, whereas embryogenesis-oriented microcalli can be moved to embryo formation medium; however, either could also proliferate callus or form embryos on the microcallus medium, depending on the genotype, source tissue, and medium composition.

Organogenesis typically relies on moving callus to a medium containing both a cytokinin and auxin or a shooting medium followed by a rooting medium. When it comes to the timeframe for regeneration, it is difficult to directly compare organogenesis and embryogenesis between different species and source tissue (Table 3). Intuitively, embryogenesis should take less time than organogenesis due to the extended time the callus needs to shoot and then root versus an embryo's ability to grow and differentiate both organs at the same time.

Embryogenic callus formation can be from somatic protoplasts (somatic embryogenesis) or from embryogenic callus-derived protoplasts (secondary embryogenesis). Embryogenesis relies on cells within the microcalli presenting embryogenic properties, *i.e.* isodiametric, cytoplasm-rich cells (Dai et al., 2010). The embryogenic microcalli can then proliferate into embryogenic callus or form embryos directly. Embryos that form from the (micro)callus can be moved to media for germination and plantlet maturation.

4.2. Rooting and Shooting Media

When it comes to regenerating plants from protoplast-derived callus, either embryogenic or somatic callus, the media composition can determine the efficiency of the regeneration. A majority of methods use solid MS media supplemented with auxin and cytokinin (Table 3). Typically, shooting is the primary goal with rooting coming shortly after, then planting in soil for maturation. It is generally easier to get roots from shoots than shoots from roots.

In cabbage leaf protoplast shoot regeneration, MS versus Gamborg B5 based media supplemented with PSK-α and with or without plant growth regulators was compared (Kiełkowska and Adamus, 2019). Microcolonies were freed from alginate layers and, after transferring to regeneration medium, the callus would turn green, remain white, or begin to brown. The browning callus was considered dead, the white callus grew slightly but did not form shoots, and the green callus led to shoot regeneration roughly 4-6 weeks after transfer. It was found that the highest shoot regeneration came from callus placed on MS media with PSK-α and without PGRs across a majority of the genotypes tested.

When determining the effect of cytokinin on shoot induction from guava (*Psidium guajava*) leaf protoplast-derived callus, BAP and kinetin concentrations were investigated (Rezazadeh and Niedz, 2015). Concentrations of 7.1 μM BAP and 11.15 μM kinetin were optimal for shoot production; a higher concentration did not significantly increase the number of shoots. It was also found that changing the kinetin level was more effective than BAP.

Some methods involve the addition of other supplements to the regeneration medium to assist the callus growth and differentiation. Activated charcoal is a common addition, with its ability to prevent browning of callus by adsorbing growth inhibitors (Prange et al., 2010a; Masani et al., 2013). Masani et al. (2013) also examined the effects of ascorbic acid to reduce discoloration and

679 promote embryogenesis. They found that ascorbic acid increased the number of embryogenic
680 calli which subsequentially improved the regeneration efficiency of oil palm embryogenic cell
681 suspension-derived protoplasts.
682
683
684 4.3. Somaclonal Variation
685
686 Somaclonal variation is the genetic or phenotypic variation that occurs in plants from tissue
687 culture. A phenotypic change can be explained by either a genetic or epigenetic modification.
688 Somaclonal variation can influence the fertility of the regenerant as well as the potential for
689 changing the ploidy level, which is crucial for breeding.
690
691 Somaclonal variation is a potential occurrence in protoplast regeneration that can reveal itself in
692 morphological or ploidy variation (Grzebelus et al., 2012; Tomiczak et al., 2015; Prange et al.,
693 2010b; Barceló et al., 2019; Sheng et al., 2011). In strawberry (*Fragaria ananassa*), morphological
694 differences between the control and regenerated protoclones were observed (including plant size
695 and leaflets per leaf) that were not explained by ploidy level changes but rather genetic variation
696 detected by microsatellite markers (Barceló et al., 2019). Prange et al. (2010b) and Tomiczak et
697 al. (2015) both collected regenerated plants that were tetraploid from protoplasts that were initially
698 diploid. In *Cyclamen coum*, it was observed that a single callus would give rise to both tetraploid
699 and diploid regenerants which was reasoned could be a result from either the chromosomes
700 doubling during callus culture or an error in separation of callus during culturing (Prange et al.,
701 2010b). With *Gentiana decumbens*, there was no morphological difference in the regenerants,
702 besides wider leaf blades (Tomiczak et al., 2015), yet 100% of the regenerated plants were
703 tetraploid.
704
705 When considering the culture method's role in this somaclonal variation, one hypothesis is that if
706 genome duplication occurred during protoplast culturing, it is most likely due to the possibility that
707 tetraploid protoplasts divide faster than diploid protoplasts, as shown in tobacco (*Nicotiana*
708 *plumbaginifolia*) (Magnien et al., 1982) and rapeseed (*Brassica napus*) (Magnien et al., 1982;
709 Chen et al., 1994). If the polyploidization occurred during callus formation, the hypothesis is
710 endoreduplication (amplification of DNA without mitosis) of callus cells, shown previously in pea
711 (*Pisum sativum*) (Ochatt et al., 2000) and barrelclover (*Medicago truncatula*) (Elmaghrabi and
712 Ochatt, 2006), and would explain this increase of DNA content. It has also been shown that plant
713 growth regulators typically added to protoplast culture media have an effect on endoreduplication
714 frequency in sugar beet (*Beta vulgaris*) (Lukaszewska et al., 2012).
715
716 Time in tissue culture increases chances of somaclonal variation. Isolating protoplast from plant
717 tissue may therefore be favorable over isolating from callus tissue in order to avoid somaclonal
718 variation due to the additional *in vitro* step that is required to obtain callus. This additional step
719 has the potential to introduce genetic variation and effect the protoplast regeneration efficiency.
720
721 While somaclonal variation is undesirable in commercial crop production, it does have the benefit
722 of creating phenotypic variability with a large number of regenerants that can be obtained through
723 protoplast regeneration. This gives the potential for the identification of mutations that could be
724 beneficial for a variety of uses, such as biotic resistance (Grzebelus et al., 2013), abiotic
725 resistance (Kiełkowska et al., 2019), or create a desirable ornamental property.
726
727

728 **5. Protoplast Transformation**
729
730 Electroporation as a method for protoplast transformation is not as popular as PEG-mediated
731 transformation. With electroporation, there are more factors to consider that potentially have
732 effects on transfection efficiency and cell survival: pulse voltage, pulse length, pulse number, cell
733 number, DNA concentration, and electroporation buffer composition (Lee et al., 2020). However,
734 when optimized, electroporation can be very efficient. Lee et al. (2020) found that when
735 electroporation transformation was optimized for cabbage protoplasts, the transformation
736 efficiency was nearly double that of PEG-mediated delivery, although both transformation rates
737 were low (3.4% and 1.8%, respectively). Wójcik and Rybczyński (2015) studied the effect of
738 electroporation the culture of embryogenic cell suspension-derived protoplasts from gentian
739 (*Gentiana kurroo*). A high electric field voltage over 1 kV/cm significantly decreased protoplast
740 survival and division. A single pulse had nine-fold higher protoplast viability than two pulses.
741 Comparing the effect of length of the electric pulse on protoplast viability, it was found that 5 ms
742 completely killed the protoplasts and 40 μs was too long and resulted in no division of the
743 protoplast. A 20 μs pulse had the highest protoplast viability and division, 70% and 44.5%
744 respectively. Significantly higher protoplast viability was obtained with an electroporation buffer
745 with KCl, higher $MgCl_2$ and pH, and lower MES (Wójcik and Rybczyński, 2015).
746
747 The more common PEG-mediated transformation requires less materials than electroporation but
748 does require chemicals that could potentially damage the protoplasts. The main factors to
749 consider with regards to transformation efficiency and cell survival are PEG concentration,
750 transfection time, DNA concentration, and cell number (which has previously been shown to
751 influence the results (Burris et al., 2016)). Transformation with PEG can reach a high
752 transformation rate, such as 90% in petunia leaf protoplasts (Subburaj et al., 2016) and 80% in
753 both wheat leaf protoplasts and rice sheath protoplasts (Shan et al., 2013). Although, a high
754 transformation rate does not translate to a large number of transformed regenerants. For
755 example, petunia leaf protoplasts transiently transformed with PEG for CRISPR/Cas9 ribonuclear
756 protein multiplexing of two genes had a 55% transfection efficiency, but only 8 of the 67
757 regenerated plants (11.9%) had indel mutations (Yu et al., 2020). PEG-mediated transformation
758 of potato (*Solanum tuberosum*) leaf protoplasts resulted in more callus formation when treated
759 with 12.5% PEG than 20% PEG; however, even the 12.5% PEG treatment resulted in a ten-fold
760 decrease in callus formation compared to the untreated control (Craig et al., 2005).
761
762
763 **6. Outlooks and Obstacles**
764
765 In our opinion, the use of protoplast regeneration in NPBT has a promising future. It has been
766 used in numerous applications of gene-editing for crop trait improvement; *e.g.* the knock-out of
767 the BRASSINOSTEROID INSENSITIVE 2 (BIN2) gene in lettuce (*Lactuca sativa*) (Woo et al.,
768 2015) and the granule bound starch synthase (GBSS) gene in potato (Andersson et al., 2018) or
769 the oligo-directed mutagenesis of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)
770 gene in flax (*Linum usitatissimum*) (Sauer et al., 2016). We expect to see many more examples
771 of its successful application in the coming years.
772
773 Nonetheless, there are obstacles that need to be addressed in order to overcome some of the
774 challenges associated with protoplast regeneration. It is a process that demands specialized
775 tissue culture expertise, requires complex manipulation, and can be time-consuming. Overall,
776 current methods for protoplast regeneration are very genotype-specific and need to be made
777 more universal for increased applicability and success.

One potential approach for making protoplast regeneration universally available is to gain fundamental knowledge of the transcriptional regulation of the regeneration process via transcriptomic analysis. While transcriptomic analysis of protoplast culture (*e.g.* in moss (*Physcomitrella patens*) protonema protoplasts for the initial 72 hours of culture (Xiao et al., 2012)) has previously been investigated, there is a lack of and difficulty in knowing the transcriptional activity of solely protoplasts destined for regeneration. Single-cell transcriptome profiling has been demonstrated (Shulse et al., 2019), but the question remains on how to differentiate between protoplasts with regeneration capability and the larger, doomed protoplast population. Additional challenges arise when taking the cell-type composition of the source organ as well as the genotype into account.

Another process that can potentially improve universal application of protoplast regeneration technologies is through ectopic expression of embryogenic or morphogenic factors. Theoretically, if an ample number of protoplasts can directly develop into embryos, the regeneration frequency would multiply, resulting in a large number of regenerated plantlets. The direct development of protoplasts into embryos could also decrease the time in tissue culture, reducing the potential of somaclonal variation. The embryogenic or morphogenic transcription factors would need be to be transiently expressed in order to avoid any developmental effects that constitutive expression may cause (*e.g.* ectopic expression of BABY BOOM causing embryogenic growth on vegetative tissue (Boutilier et al., 2002)). Identification of appropriate embryogenic or morphogenic transcription factors, which could function individually or as a collective, as well as the timing of expression would need to be investigated. Recently, a study using Arabidopsis mesophyll protoplasts showed that timed transcriptional activation of auxin biosynthesis can significantly enhance regeneration success (Sakamoto et al., 2021). It will be interesting to see whether this approach is applicable to divergent species.

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