Opinion

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Accelerating plant breeding

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The growing demand for food with limited arable land available necessitates that the yield of major food crops continues to increase over time. Advances in marker technology, predictive statistics, and breeding methodology have allowed for continued increases in crop performance through genetic improvement. However, one major bottleneck is the generation time of plants, which is biologically limited and has not been improved since the introduction of doubled haploid technology. In this opinion article, we propose to implement *in vitro* nurseries, which could substantially shorten generation time through rapid cycles of meiosis and mitosis. This could prove a useful tool for speeding up future breeding programs with the aim of sustainable food production.

Keeping up with demand

Crop production has steadily increased over time and it has been suggested that 50% of the progress is attributable to advances in crop management and breeding [1,2]. For example, the three major crops in the US, maize (*Zea mays*), wheat (*Triticum* spp.), and soybean (*Glycine max*), show positive linear increases in average yield from 1930 to 2012 [3] (Figure 1). However, changes in climatic patterns, land, and water availability now provide additional challenges for plant breeders and geneticists to ensure yield stability in varying environments [4]. To meet the projected increase of global demand for food, feed, and fiber (100% by 2050 [5]), the linear progress seen in Figure 1 will need to be increased. To increase the rate of genetic improvement (see Glossary), the efficiency, reliability, and speed of genetic improvement must be increased. In this opinion article, we propose an idea benefitting the speed of genetic improvement through the implementation of rapid generation cycling by the use of the *in vitro* nursery. Through rapid cycles of meiosis and mitosis conducted in tissue culture, generation times of crop species can be decreased allowing more opportunities for recombination and selection in a given unit of time.

The breeder's equation

Five modifiable components are used to estimate genetic gain (Box 1): additive genetic and phenotypic variance (which can be combined as narrow sense heritability), selection intensity, parental control, and time [6–9]. Choice of germplasm for formation of segregating populations affects additive variation (genetic variation that can be transmitted to the next generation), whereas choice and management of selection environments affects phenotypic variance. A combination of these components affects selection efficiency. Selection intensity, corresponding to percentage of individuals advanced after a cycle of selection, can be easily modified. The aforementioned factors can be optimized through knowledge of the germplasm and the use of predictive tools. The most critical remaining factor to maximize genetic gain is time. The number of generations per year is biologically limited. The most extreme cases are short generation times (six/year) in *Arabidopsis* (*Arabidopsis thaliana*) versus long generation times in tree species (multiple years/generation). Advances in cycle time have been limited, except for the use of off-season nurseries and doubled haploid technology.

Glossary

Backcross: a breeding methodology where a gene or few genes (e.g., resistance to a disease) usually contained within a wild or less than acceptable line are transferred to high performing lines by crossing the two lines and then repeatedly crossing the progeny back to the high performing parent while selecting for the gene or few genes of interest. The objective is to produce progeny that are as genetically similar to the high performing parent as possible while containing the gene or few genes desired from the less than acceptable parent.
BC4 line: backcross 4 line; lines which are derived after four generations of backcrossing.
Full-sib recurrent selection: a method of genotypic recurrent selection where individuals are evaluated for performance by paired plant cross pollinations which generates a set of full-sib (i.e., two shared parents) families which are tested in replicated trials to generate data for selection. Requires two seasons per cycle.
Genetic improvement/gain: the change in mean performance of a population that occurs as the result of the selection and recombination of superior performing individuals in a population.
Half-sib recurrent selection: a method of genotypic recurrent selection where individuals are evaluated for performance by cross pollination with a tester which generates a set of half-sib (i.e., one shared parent) families which are tested in replicated trials to generate data for selection. Requires one to three seasons per cycle depending on the specific method used.
Introgression: a relatively small portion of the genome of an unadapted individual, which is transferred through conventional crossing to adapted germplasm for evaluation of its utility for genetic improvement.
Linkage drag: the undesirable transfer of unwanted genes along with the gene/locus of interest due to physical linkage causing a decrease in performance of the progeny.
MABC: marker assisted backcross; a variation of the backcross breeding methodology where molecular markers are used to select for the trait of interest, and if desired for maximum recovery of the desired parent genome.
Self-incompatibility: the inability of a plant with functional male and female gametes to produce a zygote through self-fertilization.
Selfed progeny recurrent selection: a method of genotypic recurrent selection where individuals are evaluated for performance by development of selfed families (i.e., ~~F2:3, F3:4, F4:5,~~ etc.), which are tested in replicated trials to generate data for selection. Requires 3+ seasons per cycle depending on how advanced the generation of self-pollination is (i.e., more time is required for ~~F4:5 than F2:3~~).

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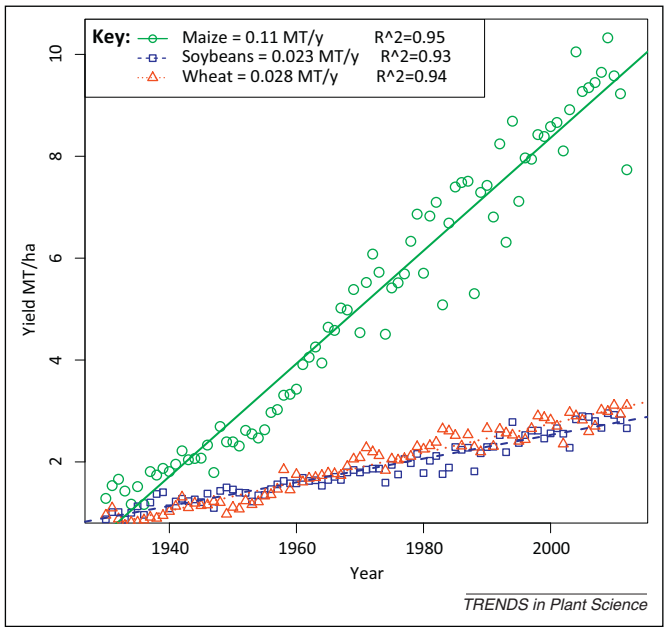

Figure 1. Yield gains of major US crops. Average yield per year in metric tons/ha (MT/ha) for each of the three major US crops (maize, wheat, and soybean) from 1930 to 2012 [3]. Each crop shows a linear increasing trend over time with maize having the highest annual gain of 0.11 MT/year followed by wheat at 0.028 MT/year and soybeans at 0.023 MT/year for average grain yield. This increase in mean yield per hectare needs to be increased to meet the demands of a growing human population.

Speeding up

Off-season nurseries, popularized by the pioneering plant breeder Norman Borlaug among others, can help to reduce the time needed to release new cultivars, for example, the time for producing a new wheat cultivar was shortened from 10–12 to 5–6 years [10]. For pure line and hybrid crop breeding, the ability to generate homozygous and homogeneous lines is another time constraint. However, by using doubled haploids (DHs) in different crop species, homozygous and homogeneous lines have been produced in two rather than five or more generations, and was the last major breakthrough to reduce cycle time [11–13]. The most popular being the maize DH system using the *R1-nj* color marker [14]. However, the different steps of the DH process (Figure 2) have biological and genotypic limitations. The success rates for haploid induction [11,15–17], adaptation to tissue culture (in the case of anther culture) [18], and doubling [19] have all been shown to be genotype-dependent in different crop species. Breeders using DHs will unintentionally practice recurrent selection for loci increasing success rates of the DH process [20], which might constrain genetic variation in breeding populations, at least for respective genome regions.

The *in vitro* nursery

Currently, the most efficient way to produce homozygous and homogeneous lines is through a combination of off-season nurseries (generations per year) and DH technology (homozygosity per generation). We propose the concept of an *in vitro* nursery, where new genotypes are formed by *in vitro* production of gametes and their subsequent fusion. Here, generation time is limited by how quickly somatic cells can form new gametes and how quickly these gametes can be fused.

Box 1. Genetic gain: the breeder's equation

The objective of plant breeding is the identification and development of superior individuals and families. The mean performance of breeding populations is increased through selection of individual plants with higher than average performance. This change in mean performance of the breeding population can be expressed as genetic gain in different forms, depending on the situation [6].

Genetic gain per cycle:

$$G_c = kch^2\sigma_P \quad [I]$$

$$G_c = \frac{kc\sigma_A^2}{\sigma_P} \quad where \quad h^2 = \frac{\sigma_A^2}{\sigma_P^2} \quad [II]$$

As seen in Equation I in the case of one cycle of selection, k is the selection differential expressed in standard deviation units, representing the percentage of individuals selected and advanced to the next generation. The degree of parental control (i.e., genetic control of males, females, both sexes) is quantified in c. Narrow sense heritability (h^2) is a measure of what proportion of phenotypic variance (σ^2_P) can be explained by additive genetic variance (σ^2_A). Equation II can be derived by substituting σ^2_A/σ^2_P for heritability. The additive genetic variance is the component of the genetic variance that is transmitted to the progeny (except in polyploids where some dominance variance is transmitted and in clonal breeding, where all genetic variance is transmitted).

Different selection schemes (e.g., half-sib, full-sib, selfed families) require different numbers of seasons to complete a full selection cycle. For comparison of alternative breeding schemes, the calculation of genetic gain per year is more informative than gain per cycle. This is achieved by dividing Equation II by the number of years (y) required per cycle.

Genetic gain per year:

$$G_y = \frac{kc\sigma_A^2}{y\sigma_P} \quad [III]$$

Equation III can be expanded further for specific situations, when different environments and replications are used and to quantify variance that is contained within and among families in the selection scheme. These expansions are beyond the scope of this article; the reader is referred to [6] for an in-depth discussion of the different forms of the genetic gain equations.

By modifying the components in Equation III, breeders are able to maximize genetic gain. Some components are simpler to manipulate than others. This article focuses on the management of time (expressed as y) as a method to maximize genetic gain.

The general progression of the *in vitro* nursery is outlined in Figure 3. Tissue is extracted from the basal leaf section of selected genotypes and converted into an *in vitro* cell culture and induced to mitotically divide through application of growth regulators such as ~~2–4D~~ [21], which can be maintained in minimal space requirements in a laboratory setting with each cell callus occupying approximately 4 mm^2 [22]. Genotypes of interest are subsequently isolated and single somatic cells are induced to undergo meiosis for generation of new gametes. These gametes are subsequently fused to generate new genotypes in a similar way to the *in vivo* unification of pollen and egg cells. However, in contrast to the *in vivo* system, where the breeder would need to wait until seed maturity and the flowering of progeny to produce the next generation, fused diploid cells could immediately be induced to undergo meiosis within the *in vitro* system, and produce gametes for new crosses, or for artificial genome doubling to produce a new homogeneous/homozygous cell line [23]. Several techniques exist for fusion of plant gametes *in vitro*: electrically induced fusion, chemically induced fusion, and calcium induced fusion [24,25]. Successful fusion of plant gametes *in vitro* has been reported

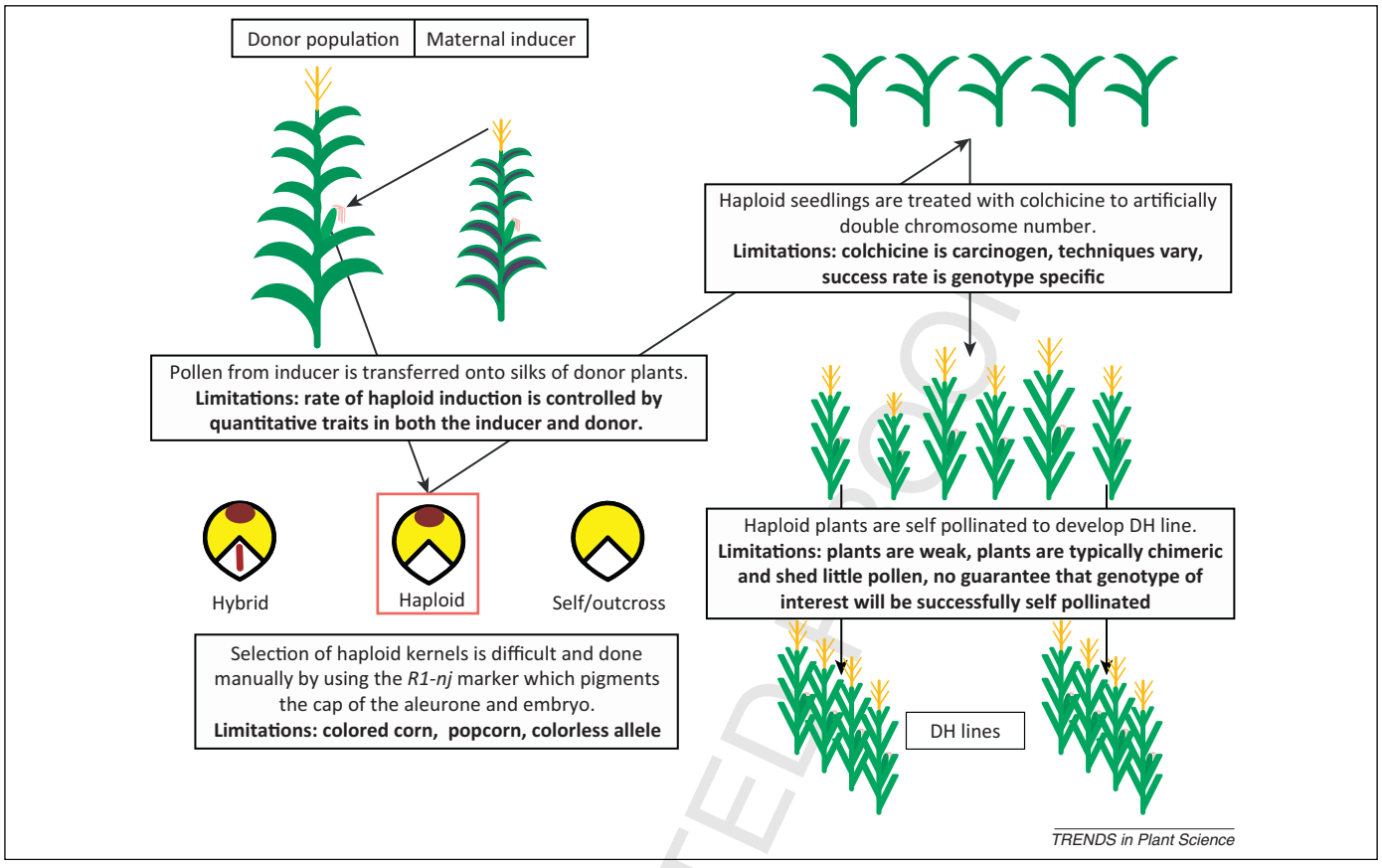

Figure 2. The maize doubled haploid system. Maize doubled haploid (DH) technology is a specific example of DH technology used with great success by public and private plant breeders to shorten the time it takes to generate a homozygous line from eight to two seasons. This is arguably the latest major breakthrough in cycle time (see Box 1 for a reduction in years per cycle). Although DH technology is used with success in maize and other crop species, there are limitations (as noted in the figure). The rate of haploid induction is genetically controlled by quantitative trait loci (QTL) in both the inducer and donor population. The *R1-nj* [14] marker allele used to identify haploid kernels is useless, if the kernels are colored or if they carry the colorless allele. Rates of doubling in haploid plants are typically low and highly dependent on both technique and genotype. The doubling agent, colchicine, is a carcinogen. Those plants that successfully double their genomes typically shed little pollen and there is no guarantee that the optimal genotype will set seed and advance to testing. For now, the benefits of time savings outweigh the drawbacks.

for maize [23,26], wheat [27], rice (*Oryza sativa* L.) [28], and tobacco (*Nicotiana tabacum* L.) [29]. The main biological bottlenecks are now limited to the induction of meiosis and the rate of cell division, whose estimation is critical to successful tissue culture [22]. It is estimated that plant cell division rates can range from 22 to 48 h [21].

This entire process would need to be coupled with marker-based and/or genomic selection. Evaluation and selection within the *in vitro* nursery would be accomplished by running marker analyses on new cell lines and/or gametes. Time can be saved by using single cells for whole genome amplification and subsequent marker analysis [30,31]. Selection efficiency can be increased by selecting gametes versus zygotes. In traditional breeding practices, selection is limited to the diploid (or polyploid) plant in most cases. A notable exception would be selection on haploid plants in a DH system. In the *in vitro* system, specific and targeted matings could be achieved through mitotic division of gametes and subsequent marker analysis for genomic gamete selection (GGS). Although no examples exist of the mitotic division and callus formation of artificially induced gametes, other biological examples such as yeast, the ability to grow haploid callus in anther culture, and the normal (although weak) functionality of haploid maize plants provide evidence that this is possible. These haploid mitotic divisions allow for the selection of gametes without their destruction. This could also be coupled with optimization procedures for generating optimal genotypes with minimal numbers of resources and time [32] increasing selection efficiency. Selected cell lines could then be converted to mature plants, which can be used for phenotypic evaluation. In maize, converting cell lines into mature plants will be the most time-demanding step, currently requiring 148 to 215 days from gamete fusion to the harvest of mature seed. Plant regeneration is not 100% efficient and varies in different species with percentages reported as 37–73% in tobacco, 25–48% in rice, 41–59% in maize, 5–33% in cotton (*Gosyppium hirsutum*), and 93–100% in soybean [33]. This step is probably also genotype-dependent and warrants more research into the regeneration of plants from tissue culture.

The obvious advantage of this system is the reduction in time for line development. With a conservative estimate for a division rate of 48 h/cycle, a new cycle could be generated every week, provided that marker analyses could be conducted at a similar pace. For comparison, a DH line can be produced in 1 year with only a single recombination event. Alternatively, in the same time period, a line produced from the *in vitro* nursery could result from 12 cycles of recombination and selection (at 1 week intervals), assuming that meiotic induction and division takes 48 h, similar to the division rate for mitosis, before plant regeneration is

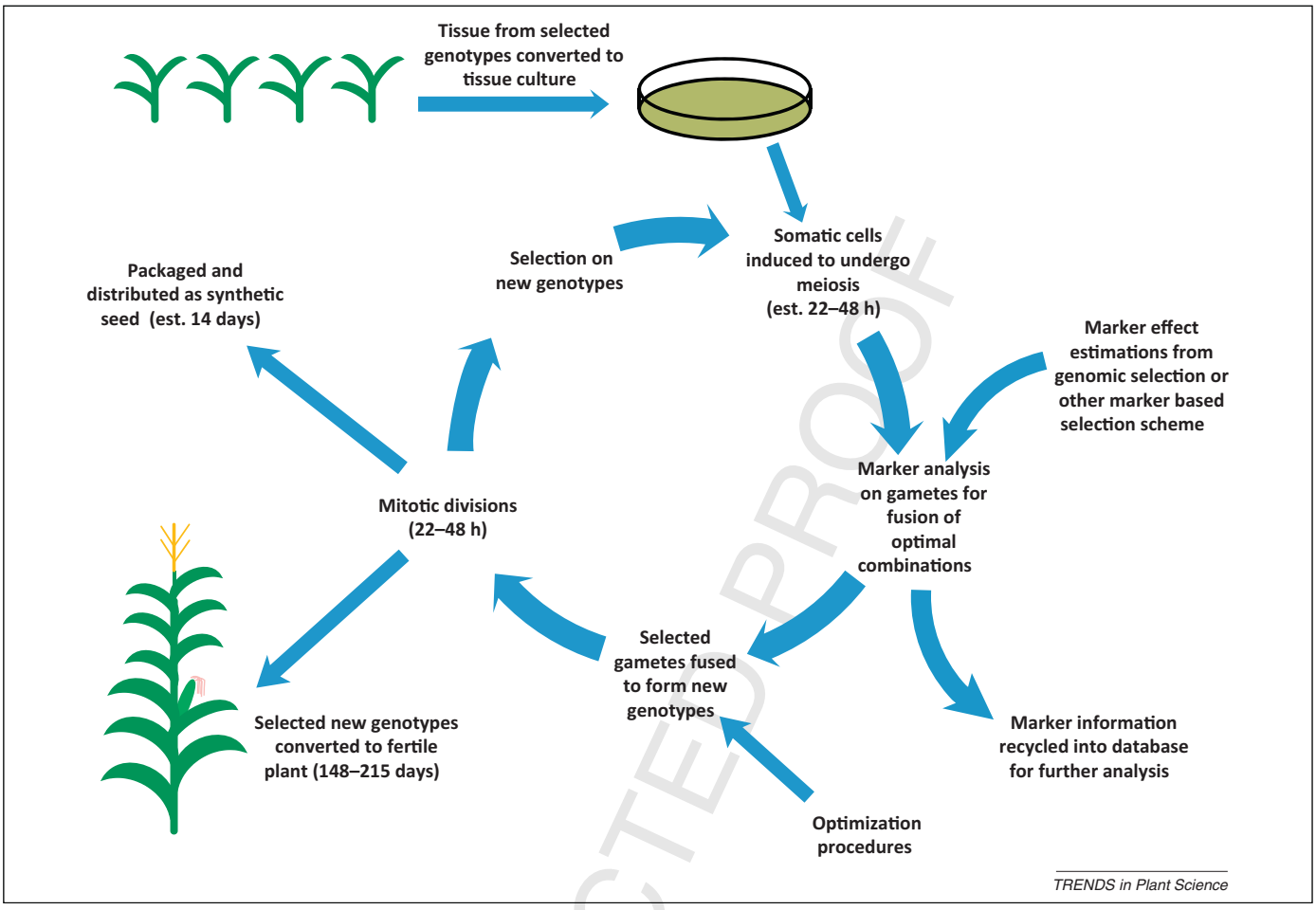

Figure 3. The *in vitro* nursery. The general scheme of the *in vitro* nursery. First, tissue from selected genotypes must be extracted and converted into a tissue culture. A genotype dependency for tissue culture conversion and success is likely. Once the somatic cells have stabilized in culture, they are induced to undergo meiosis. After gametes are formed, they are allowed mitotic cycles which lead to clonal cells, so that DNA can be extracted from some of those cells for marker analyses. Marker effect estimation based on genomic selection, marker-assisted backcrossing, or marker-assisted selection are incorporated. Optimization procedures can then be incorporated to make the stacking of optimal loci as efficient as possible. Optimal gametes are then selected and fused to form a new diploid individual. Mitotic divisions are required to enable DNA extraction. At this junction, selected new genotypes can either be converted into fertile plants or into synthetic seeds for phenotypic evaluation. The cell line can then be immediately recycled in the nursery and induced to form new gametes to complete the cycle.

limiting seed production. The utility of *in vitro* nurseries is obvious for both mapping and marker-assisted backcrossing (MABC). Mapping experiments require the development of large (i.e., >200 families) populations, which can be used for genotyping and phenotyping. Development and maintenance of large populations require significant resources including both labor force and field space. This is particularly true for species with large generation time and space requirements. The *in vitro* nursery system could allow for the quick and efficient development of cell lines that can be subsequently stored and/or converted into plants to be used for phenotyping and/or production.

In MABC, the ultimate goal is to transfer a gene of interest into an existing cultivar/line. One main challenge of MABC is to remove unfavorable alleles of closely linked genes, that is, to eliminate linkage drag, particularly in the case of exotic introgressions. Thus, multiple individuals need to be evaluated, which is costly and requires a significant amount of resources. MABC programs could alternatively be conducted within *in vitro* nurseries. Large numbers of individuals could be generated within a controlled laboratory setting and evaluated using markers. This would allow rapid and efficient introgression of genes of interest. The utility of this system becomes increasingly superior, as the number of loci to be introgressed increases [13].

Another application of an *in vitro* nursery would be to overcome self-incompatibility (SI), which is present in many cultivated species [34–37]. To successfully produce single cross hybrids in SI crops, breeders must be able to generate homogeneous and homozygous parental inbred lines to produce the hybrid. The generation of these inbred lines is impossible in the case of SI. This process, however, occurs through the interaction of pollen tubes with stigma [38]. In the *in vitro* nursery, this pollination stage can be bypassed and gametes can be fused directly, thus overcoming the issue of SI. We envisage a system, where somatic cells of these species are used to generate gametes which could be subsequently fused with gametes from the same cell (simulating self-pollination) or artificially doubled simulating the DH process to generate homozygous and homogeneous lines that can be subsequently used to generate hybrids. This idea can be taken one step further. Gametes from selected homozygous and homogeneous lines could be fused *in vitro* to generate zygotes which are the desired hybrid combination. This process could be combined with the development of synthetic seeds

where somatic embryos are encapsulated to form an artificial seed, which can be packaged and distributed to growers similar to a normal seed. Successful germination of an artificial seed generated from somatic tissue has been demonstrated in species such as alfalfa (*Medicago sativa*) [39], cyclamen (*Cyclamen persicum* Mill.) [40], and salparni (*Desmodium gangeticum* L.) [41].

The utility of this system is more beneficial for plant species with long generation times such as those of the genus Leucaena, which can take up to 2 years to flower [42], pecan (*Carya illinoinensis*) which flower at 6–7 years of age [43], and other woody species. However, its utility could also extend to species which normally require vernalization or a chilling cycle to induce flowering such as peaches (*Prunus persica*) [44], and wheat [45] as a method to overcome these requirements and produce new sexual progeny at any time in the year. Apomictic species for which recovery of sexually generated populations to be used as variation for selection is difficult may also benefit such as those of citrus species [46]. Finally, annual crops, such as maize and soybean, could also benefit through rapid generation of new populations for selection and line conversion. For example, consider the time and expenses used to convert new breeding lines of maize and soybean into those which contain desirable genes for resistance to a pathogen or transgenes. This process which works in tandem with line development can require up to six seasons to produce a suitable BC4 line and assuming three seasons per year would take 2 years to complete. Using the proposed *in vitro* nursery, this process could be shortened to 257 days assuming 1 week per cycle and 215 days to regenerate a fertile plant. The savings will not only be in time but also in cost of land, seed shipment to off-season nurseries, labor, and a smaller number of lines converted.

Concluding remarks and future challenges

The purpose of this manuscript is to combine recent advances in different fields of biology and conceptualize a technique that could substantially advance efficiency of plant breeding, once becoming available. The idea of an *in vitro* nursery presented in the previous sections, although new and innovative, does have obvious problems and gaps at present. The first, and most important, is the ability to stimulate meiosis and to generate gametes *in vitro*. Recent advances in both plant and animal models provide insight into gamete formation *in vitro*. For animals, the production of egg cells *in vitro* has been reported [47], as well as the successful production of artificial gametes in mice [48]. The first study required the use of stem cells, whereas the second used testicular tissue and thus did not induce gametes from purely somatic cells. A recent review [49] outlined the current advances in the development of artificial gametes in animals and the significant obstacles that remain. The authors noted that the knowledge needed to generate functional germ cells *in vitro* exists but the methodology is in its infancy [49]. In contrast to animals, whose germ lines are established early in development, plants specify germ lines later in development and can have multiple germ lines [50]. For example, a hypoxic environment causes any cell in an early maize anther to convert to a germ cell [51]. It is currently unclear if recombination is occurring, although it is likely because meiosis is induced, and more research is needed to confirm this. The genetic mechanisms which underlie the control of plant meiosis are being elucidated and research is ongoing with practical applications, including the *in vitro* nursery, across the plant sciences. The complexity and breadth of the research in this field is beyond the scope of this manuscript, but the reader is referred to [52] for an up-to-date description of the latest breakthroughs. This provides an initial framework for producing gametes *in vitro* from somatic cells. Similar to most techniques in biology, it is likely that this process will not be 100% efficient. Therefore, there would be a need to distinguish between haploid and diploid cells, which may not be trivial.

The use of the *in vitro* nursery will also require the continued advancement of predictive tools that can be used in genomic selection schemes. This research is not specific to applications for the *in vitro* nursery as it would also assist current breeding programs.

An array of issues still remains with this proposed idea. Growing cells in tissue culture can generate genotype dependencies [53–55] and the use of the *in vitro* nursery will cause unintended selection for loci, which control success of cell culture. Genotype dependency of regeneration is the major challenge in tissue culture techniques [56–58]. However, genes or quantitative trait loci (QTL) for regeneration in tissue culture have been identified [59,60] and can help to overcome this bottleneck. Recent reports show that targeting young zygotes or isolating cells during the early callus phase for plant regeneration has less genotype dependency than those which are allowed to go through a callus growth phase and are then regenerated [61–63]. Another issue is the phenomenon of somaclonal variation. When plants are grown *in vitro*, stress induces changes in regenerated plants. Somaclonal variation can provide useful variation [64]. In the *in vitro* nursery changes due to somaclonal variation, such as activation of transposable elements, can counteract the generation of homogeneous and homozygous lines.

Despite these challenges, a major benefit would be a larger number of generations per year with the potential to increase the rate of genetic gain, which may, in turn, increase the rate at which the mean yield of crops improves (Figure 1).

References

1 Duvick, D.N. (2005) The contribution of breeding to yield advances in maize (*Zea mays* L.). In *Advances in Agronomy* (Donald, L.S., ed.), pp. 83–145, Academic Press
2 Lopes, M.S. *et al.* (2012) Genetic yield gains and changes in associated traits of CIMMYT spring bread wheat in a "historic" set representing 30 years of breeding. *Crop Sci.* 52, 1123–1131
3 United States Department of Agriculture-National Agricultural Statistics Service (USDA-NASS) (2013) *Field Crops Statistics*, USDA-NASS
4 Brummer, E.C. *et al.* (2011) Plant breeding for harmony between agriculture and the environment. *Front. Ecol. Environ.* 9, 561–568
5 Tilman, D. *et al.* (2011) Global food demand and the sustainable intensification of agriculture. *Proc. Natl. Acad. Sci. U.S.A.* 108, 20260–20264
6 Fehr, W.R. (1993) *Principles of Cultivar Development*, McGraw-Hill
7 Bernardo, R. (2010) *Breeding for Quantitative Traits in Plants*, Stemma Press

8 Falconer, D.S. and Mackay, T.F.C. (1996) *Introduction to Quantitative Genetics*, Pearson
9 Hallauer, A.R. *et al.* (2010) *Quantitative Genetics in Maize Breeding*, Springer
10 Borlaug, N.E. (2007) Sixty-two years of fighting hunger: personal recollections. *Euphytica* 157, 287–297
11 Ferrie, A.M.R. and Möllers, C. (2010) Haploids and doubled haploids in *Brassica* spp. for genetic and genomic research. *Plant Cell Tissue Organ Cult.* 104, 375–386
12 Geiger, H.H. (2009) Doubled haploids. In *Handbook of Maize* (Bennetzen, J.L. and Hake, S., eds), pp. 641–657, Springer
13 Lübberstedt, T. and Frei, U.K. (2012) Application of doubled haploids for target gene fixation in backcross programmes of maize. *Plant Breed.* 131, 449–452
14 Nanda, D.K. and Chase, S.S. (1966) An embryo marker for detecting monoploids of maize (*Zea mays* L.). *Crop Sci.* 6, 213–215
15 Prigge, V. *et al.* (2011) Doubled haploids in tropical maize: I. Effects of inducers and source germplasm on in vivo haploid induction rates. *Crop Sci.* 51, 1498–1506
16 Prigge, V. *et al.* (2012) New insights into the genetics of in vivo induction of maternal haploids, the backbone of doubled haploid technology in maize. *Genetics* 190, 781–793
17 Kebede, A.Z. *et al.* (2011) Effect of source germplasm and season on the in vivo haploid induction rate in tropical maize. *Euphytica* 180, 219–226
18 Germanà, M.A. (2010) Anther culture for haploid and doubled haploid production. *Plant Cell Tissue Organ Cult.* 104, 283–300
19 Castillo, A.M. *et al.* (2009) Chromosome doubling in monocots. In *Advances in Haploid Production in Higher Plants* (Touraev, A. *et al.*, eds), pp. 329–338, Springer
20 Prigge, V. and Melchinger, A. (2012) Production of haploids and doubled haploids in maize. In *Plant Cell Culture Protocols* (Loyola-Vargas, V.M. and Ochoa-Alejo, N., eds), pp. 161–172, Humana Press
21 Bhojwani, S. and Dantu, P. (2013) Tissue and cell culture. In *Plant Tissue Culture: An Introductory Text*. pp. 39–50, Springer
22 Godoy-Hernández, G. and Vázquez-Flota, F. (2012) Growth measurements: estimation of cell division and cell expansion. In *Plant Cell Culture Protocols* (Loyola-Vargas, V.M. and Ochoa-Alejo, N., eds), pp. 41–48, Humana Press
23 Kranz, E. and Lorz, H. (1993) *In vitro* fertilization with isolated, single gametes results in zygotic embryogenesis and fertile maize plants. *Plant Cell* 5, 739–746
24 Kranz, E. and Scholten, S. (2008) *In vitro* fertilization: analysis of early post-fertilization development using cytological and molecular techniques. *Sex. Plant Reprod.* 21, 67–77
25 Bhojwani, S. and Dantu, P. (2013) *In vitro* pollination and fertilization. In *Plant Tissue Culture: An Introductory Text*. pp. 155–171, Springer
26 Faure, J-E. *et al.* (1994) An *in vitro* system for adhesion and fusion of maize gametes. *Science* 263, 1598–1600
27 Ponya, Z. *et al.* (2004) Structural change in the endoplasmic reticulum during the in situ development and *in vitro* fertilisation of wheat egg cells. *Sex. Plant Reprod.* 17, 177–188
28 Uchiumi, T. *et al.* (2007) Establishment of an *in vitro* fertilization system in rice (*Oryza sativa* L.). *Planta* 226, 581–589
29 Sun, M-X. *et al.* (2001) *In vitro* double fertilization in *Nicotiana tabacum* (L.): the role of cell volume in cell fusion. *Sex. Plant Reprod.* 13, 225–229
30 Woyke, T. *et al.* (2011) Decontamination of MDA reagents for single cell whole genome amplification. *PLoS ONE* 6, e26161
31 Treff, N.R. *et al.* (2010) Accurate single cell 24 chromosome aneuploidy screening using whole genome amplification and single nucleotide polymorphism microarrays. *Fertil. Steril.* 94, 2017–2021
32 Xu, P. *et al.* (2011) An optimization approach to gene stacking. *Eur. J. Oper. Res.* 214, 168–178
33 Greenway, M.B. *et al.* (2012) A nutrient medium for diverse applications and tissue growth of plant species *in vitro*. *In vitro Cell. Dev. Biol. Plant* 48, 403–410
34 Iwano, M. and Takayama, S. (2012) Self/non-self discrimination in angiosperm self-incompatibility. *Curr. Opin. Plant Biol.* 15, 78–83
35 Sun, P. and Kao, T.H. (2013) Self-incompatibility in *Petunia inflata*: the relationship between a self-incompatibility locus F-box protein and its non-self S-RNases. *Plant Cell* 25, 470–485
36 Chantha, S-C. *et al.* (2013) Secondary evolution of a self-incompatibility locus in the Brassicaceae genus leavenworthia. *PLoS Biol.* 11, e1001560
37 De Nettancourt, D. (1972) Self-incompatibility in basic and applied researches with higher plants. *Genet. Agr.* ~~1–2~~, 163–216
38 Kaothien-Nakayama, P. *et al.* (2010) Self-incompatibility systems in flowering plants. In *Plant Developmental Biology – Biotechnological Perspectives* (Pua, E.C. and Davey, M.R., eds), pp. 459–485, Springer
39 Liu, W. *et al.* (2013) Preservation and faithful expression of transgene via artificial seeds in alfalfa. *PLoS ONE* 8, e56699
40 Winkelmann, T. *et al.* (2004) Germination of encapsulated somatic embryos of *Cyclamen persicum*. *Hort. Sci.* 39, 1093–1097
41 Puhan, P. and Rath, S.P. (2012) Induction, development and germination of somatic embryos from *in vitro* grown seedling explants in *Desmodium gangeticum* L.: a medicinal plant. *Res. J. Med. Plant* 6, 346–369
42 Brewbaker, J.L. (2008) Registration of KX2-Hawaii, interspecific-hybrid leucaena. *J. Plant Reg.* 2, 190
43 Thompson, T. and Conner, P. (2012) Pecan. In *Fruit Breeding* (Badenes, M.L. and Byrne, D.H., eds), pp. 771–801, Springer
44 Byrne, D. *et al.* (2012) Peach. In *Fruit Breeding* (Badenes, M.L. and Byrne, D.H., eds), pp. 505–569, Springer
45 Chen, A. and Dubcovsky, J. (2012) Wheat tilling mutants show that the vernalization gene *vrn1* down-regulates the flowering repressor *vrn2* in leaves but is not essential for flowering. *PLoS Genet.* 8, e1003134
46 Ollitrault, P. and Navarro, L. (2012) Citrus. In *Fruit Breeding* (Badenes, M.L. and Byrne, D.H., eds), pp. 623–662, Springer
47 Cheloufi, S. and Hochedlinger, K. (2012) Reproductive biology: stem cells bear eggs. *Nature* 491, 535–536
48 Sato, T. *et al.* (2011) *In vitro* production of functional sperm in cultured neonatal mouse testes. *Nature* 471, 504–507
49 Kashir, J. *et al.* (2012) Viability assessment for artificial gametes: the need for biomarkers of functional competency. *Biol. Reprod.* 87, 114
50 Whipple, C. (2012) Defining the plant germ line – nature or nurture? *Science* 337, 301–302
51 Kelliher, T. and Walbot, V. (2012) Hypoxia triggers meiotic fate acquisition in maize. *Science* 337, 345–348
52 Wijnker, E. and Schnittger, A. (2013) Control of the meiotic cell division program in plants. *Plant Reprod.* 26, 143–158
53 Karp, A. (1994) Origins, causes and uses of variation in plant tissue cultures. In *Plant Cell and Tissue Culture* (Vasil, I. and Thorpe, T., eds), pp. 139–151, Springer
54 Sharma, V. *et al.* (2004) A highly efficient plant regeneration system through multiple shoot differentiation from commercial cultivars of barley (*Hordeum vulgare* L.) using meristematic shoot segments excised from germinated mature embryos. *Plant Cell Rep.* 23, 9–16
55 Simmonds, D.H. and Donaldson, P.A. (2000) Genotype screening for proliferative embryogenesis and biolistic transformation of short-season soybean genotypes. *Plant Cell Rep.* 19, 485–490
56 Anami, S. *et al.* (2010) Somatic embryogenesis and plant regeneration of tropical maize genotypes. *Plant Cell Tissue Organ Cult.* 102, 285–295
57 Khan, T. *et al.* (2010) High-frequency regeneration via somatic embryogenesis of an elite recalcitrant cotton genotype (*Gossypium hirsutum* L.) and efficient Agrobacterium-mediated transformation. *Plant Cell Tissue Organ Cult.* 101, 323–330
58 Targońska, M. *et al.* (2013) The specificity and genetic background of the rye (*Secale cereal* L.) tissue culture response. *Plant Cell Rep.* 32, 1–9
59 Krakowsky, M.D. *et al.* (2006) Quantitative trait loci for callus initiation and totipotency in maize (*Zea mays* L.). *Theor. Appl. Genet.* 113, 821–830
60 Nishimura, A. *et al.* (2005) Isolation of a rice regeneration quantitative trait loci gene and its application to transformation systems. *Proc. Natl. Acad. Sci. U.S.A.* 102, 11940–11944
61 Holme, I. *et al.* (2012) Transformation of barley (*Hordeum vulgare* L.) by *Agrobacterium tumefaciens* infection of *in vitro* cultured ovules. In *Transgenic Plants* (Dunwell, J.M. and Wetten, A.C., eds), pp. 151–161, Humana Press
62 Parmar, S. *et al.* (2012) Plant regeneration from mature embryo of commercial Indian bread wheat (*Triticum aestivum* L.) cultivars. *Physiol. Mol. Biol. Plants* 18, 177–183
63 Ebrahimie, E. *et al.* (2006) Direct shoot regeneration from mature embryo as a rapid and genotype-independent pathway in tissue culture of heterogeneous diverse sets of cumin (*Cuminum cyminum* L.) genotypes. *In vitro Cell. Dev. Biol. Plant* 42, 455–460
64 Bhojwani, S. and Dantu, P. (2013) Somaclonal variation. In *Plant Tissue Culture: An Introductory Text*. pp. 141–154, Springer