Placement Report

Plant breeding with the aid of biotechnology at CN Seeds Ltd.

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Executive Summary

This report summarises the four biotechnology projects I was involved in during my year placement at CN Seeds Ltd. CN Seeds is a family-run company specialising in the breeding of commercial herb, babyleaf salad and oriental vegetable seeds for the UK and global market. Biotechnology was used to overcome problems encountered in plant breeding projects. Anther culture was investigated as a means of generating double haploid plants which would avoid the problem of trait segregation. Leaf disc culture was trialled with the aim of duplicating a nuclear male sterile plant without undergoing sexual reproduction, which would result in some fertile offspring. Though this technique was not totally successful, the research is still ongoing. Meristem tissue culture was a means of generating a large identical population without encountering the problems of inbred depression and trait segregation. Finally, embryo rescue tissue culture was trialled successfully, generating interspecific hybrids for current breeding programs which were previously unobtainable. Overall the projects were successful and they are now either offering immediate tools to help overcome problems encountered in breeding projects or will do so in the near future. The improved outcomes and speed of plant breeding with biotechnological aids means that CN Seeds will have greater chances of producing market leading varieties in the future.

Profile of CN Seeds Ltd.

CN Seeds Ltd. is a family-owned business that has been running since 1990 and is based in Pymoor, Cambridgeshire, UK. CN Seeds specialises in the breeding and sale of babyleaf salad, herb and oriental vegetable seeds. Seed sales are global, with large markets in Spain, America, Norway, and the UK.

There is a growing demand for new and novel leafy products especially amongst the affluent younger generations from Western countries, who are influenced by healthy living trends (Anderson 2015, FLIC, 2014). For instance, kale, with many acclaimed health benefits (Ware 2015), has been sold greatly in recent years to America for babyleaf salad production, and now the trend is catching on in Australia and the UK (Langley 2013; Straight 2014). CN Seeds has grown rapidly over the last decade due to its ability to respond and predict the frequent market changes and developments.

CN Seeds breeds its own commercial lines of herbs, salad crops and oriental vegetable types, with the initial breeding taking place at its base in Pymoor. Each breeding line is bred with aims of improving or introducing certain desirable characteristics. Pre-commercial trials of the potentially commercial varieties will be conducted in a range of growing conditions to compare the performance of the lines prior to commercialisation. CN Seeds crop seed in different locations and countries, depending on what best suits the crop in question, before shipping the seed back to Pymoor where it can be graded and treated as necessary. CN Seeds has a knowledgeable team and specialist machinery for the grading, sorting and treating of seeds, which helps to ensure a uniform and disease-free crop.

The plant breeders at CN Seeds are constantly trying to improve and develop commercial lines which will be best suited to the market. Certain traits are of interest to the grower such as disease resistance, which has many benefits such as reducing the crop protection costs, and likely reducing chemical residues on the crop and therefore improving consumer safety. There are also cosmetic traits of interest, such as improved leaf colour and novel leaf shape. Along with the breeding lines, CN Seeds also has exclusive rights to sell some commercial varieties.

The company itself is reasonably small with around 20 employees, though it is constantly expanding. Beneath the director and managing director there are smaller areas of expertise including sales, quality control, stock control, seed treatment, seed transport, plant breeding and commercial trialling. Although small compared to its' competitors CN Seeds exerts significant dominance on the market with leading market varieties such as coriander Cruiser. CN Seeds is constantly reinvesting in its business with regards to improved technological equipment and expertise, allowing for improved plant breeding and seed treatment. This should reap circular rewards for the company who should be able to expand into new markets and increase their customer base in the coming years.

Introduction

During my time at CN Seeds as an assistant plant breeder, I spent a significant amount of time in the laboratory using biotechnological techniques to compliment traditional plant breeding methods. Biotechnology can be used to over come bottlenecks encountered in plant breeding, without such techniques these obstacles may not be overcome or a similar outcome may take a significantly longer amount of time to achieve. These biotechnological practices should considerably shorten breeding from 7 to 1 years in some cases (Chawla 2002; Shimelis and Laing 2012).

Plant breeding involves the artificial selection and crossing of plants with desirable characteristics. Natural genetic recombination facilitates traditional breeding methods, it takes many generations to transfer and stabilise genes within a breeding line (Sleeper and Poehlman 2006). The tissue culture methods used in the laboratory involved taking explants, differentiated tissue or organs, and putting them into a nutrient medium in the hope that the cells would regenerate into a new whole plant by firstly dedifferentiating into meristematic cells. These techniques have been used for decades with much success (Smith and Drew 1990). With these breeding methods genetic change is due to natural genetic recombination, these techniques are not considered to be genetic modification whereby specific pieces of DNA are taken or introduced to the nucleus (Gasser and Fraley 1989).

A range of species are currently being bred in different breeding programs, therefore the protocol for the traditional breeding and biotechnological methods need to be adapted. As many species that are being bred at CN Seeds have had little breeding development, the literature and protocols published is limited; compared for instance to Beta vulgaris (common sugar beet) which has been extensively researched and bred and has a significant amount of published data available making future research easier. For plants with little research new protocols need to be developed by adapting general protocols of similar species.

Here I will report on the main biotechnological projects in the laboratory, including a critical evaluation of the projects success and application, and how they will impact upon future plant breeding within the company.

Anther tissue culture

One of the first biotechnological techniques I learnt in the laboratory was anther culture. Anther culture is used by plant breeders to gain a haploid plant. The plantlet being haploid, having originated from gametophyte microspores, offers many advantages to plant breeding. Duplication of the chromosomes to a diploid plant means that both chromosomes are identical, this eliminates the time consuming process of selfing plants for many generations (Palmer et al. 2005). When crossing double haploid plants there is no risk of segregation of traits, such as leaf colour, which removes this risk of genetic breakdown of the crop in successive generations. Some traits themselves are difficult to maintain without anther culture as they are the result of recessive alleles; recessive alleles are often masked in diploid plants by their countering

dominant allele (Tadesse et al. 2003). In principle the tissue culture should follow the basic developmental stages pictured in figure 1.

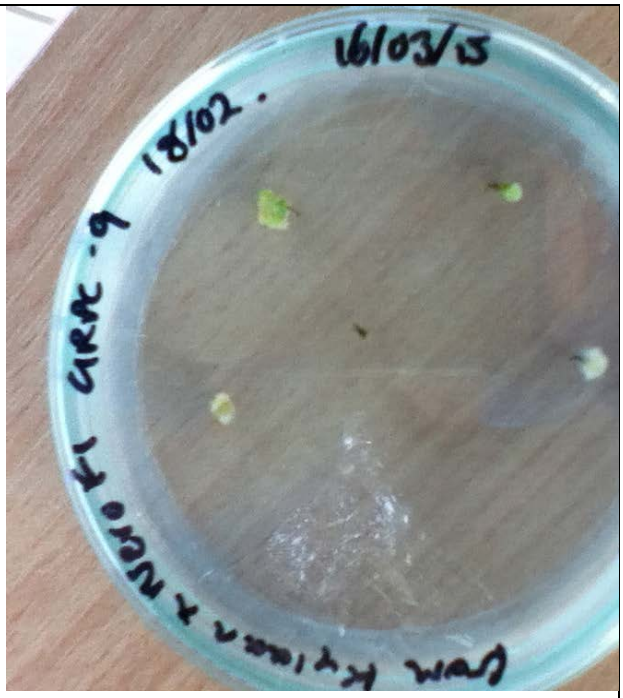
At CN Seeds it was desirable to have a anther culture protocol for our breeding lines, so that we could select haploid plants with desired characteristics and breed from these without incurring trait segregation. Trait segregation can severely de-value a commercial crop as varied characteristics will fail to meet crop specifications. The ability to perform anther culture would be of huge financial and competitive advantage, as it would shorten the breeding time from around 7 to 1 years (Chawla 2003).

We were trying to develop and finalise an anther culture protocol for different species and genotypes which are currently bred at CN Seeds. Firstly I found that there was an optimum anther developmental stage, when there was the greatest callus induction from anthers, generally medium sized buds were the most successful, though this varied depending on the species. There was also a critical time and temperature to take the buds from the mother plant for culturing, if the donor plants were in a too warm and humid climate the buds would open meaning the anthers would be unprotected from sterilisation procedures. We found that some genotypes were more responsive and had a much higher callus induction rate, figure 2. The percentage of anthers with callus growth induced was as high as 76%, which is a very high percentage (Machii et al. 1998). Over 50% of the explants had callus induction, a high percentage considering the experimental stage of this procedure.

Initially we did struggle to stimulate some genus into the shooting phase of organogenesis, as the calli rooted prior to shooting. Here we trialled many medias with differing hormones, particularly increased cytokinin ratios. However the best results were found when the calli were placed in a hormone free media for two or more weeks to starve them of the endogenous auxin concentrations, commonly found that some genus have their own endogenous hormone supplies (Dunwell 1981). Shooting organogenesis then occurred in a high cytokinin media.

Overall we had very good callus induction in a range of genus. Though we had a problem of premature rooting prior to shooting organogenesis, we were able to manipulate the protocol to overcome this. Overcoming this problem was a great success and took a lot of patience. We have collected a lot of data on the different genotype responses, which maybe used in the future when trying to fix a new trait of interest in a breeding line using anther culture.

Excised anther on callus induction media



Callus growing from anthers

Shooting organogenesis from undifferentiated callus

Plantlet hardening off in cotton wool and reduced humidity

Figure 1. Pictogram of the developmental stages of anther culture.

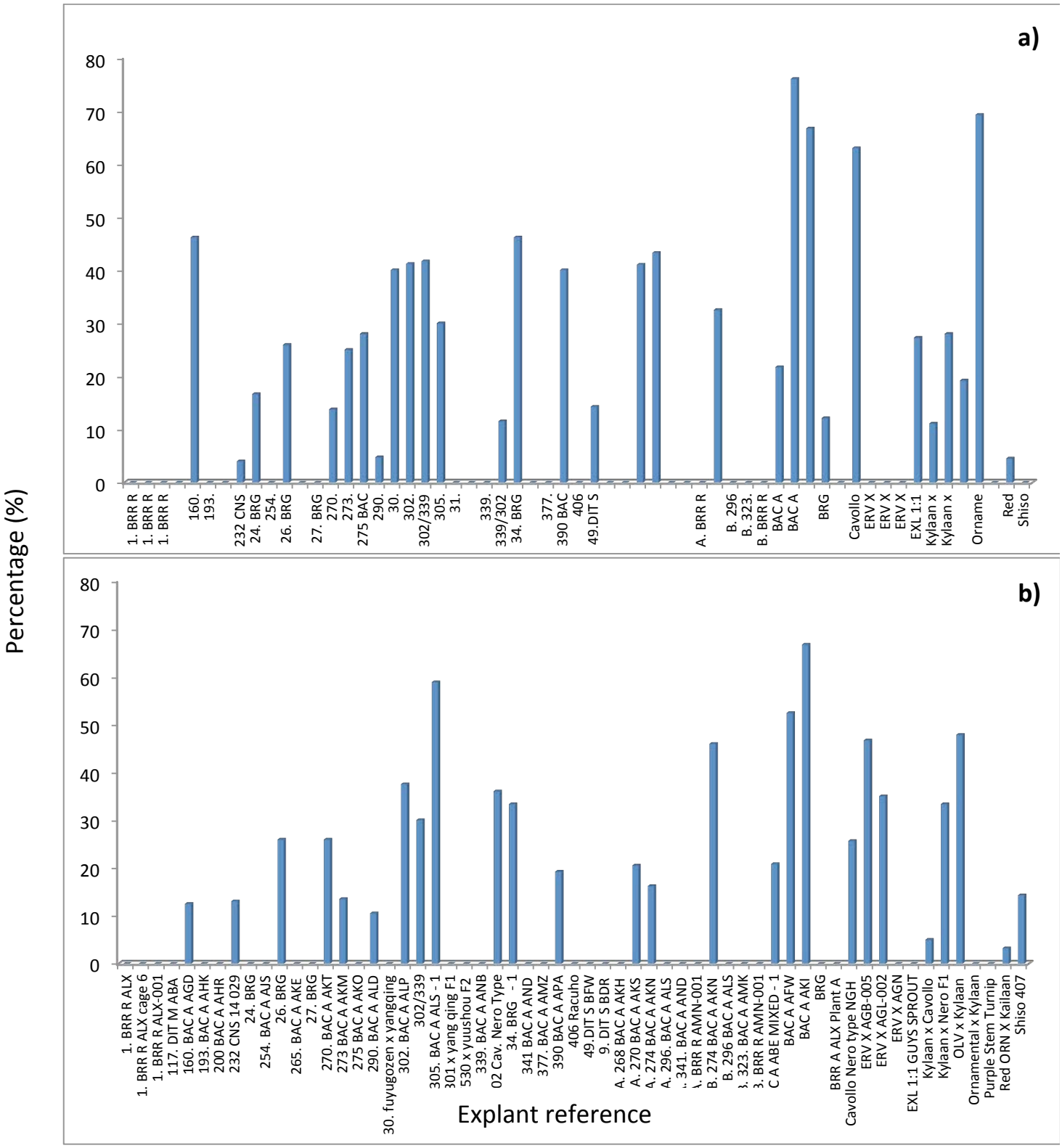


Figure 2. The percentage of anthers which had callus growth induced, callus induction recorded from different explants. a) Percentage of anthers with callus induced in trialled induction media CIRPC-9. b) Percentage of anthers with callus induced in trialled induction media CIRPC-10.

Leaf disc tissue culture

Leaf disc culture is a technique for duplicating the mother plant many times over, this can be useful if genetic traits are lost when multiplying populations from seeds. We had a particular project of interest for this application, where one of our breeding lines had nuclear male sterility (NMS). NMS will allow the production of hybrid seed, as the NMS plants will be sterile males, and therefore only act as female plants. NMS is difficult however to maintain through seed cycles as the responsible genes are often recessive and there are no restorer plants found in the wild, therefore the offspring will segregate between being fertile and NMS (Budar and Pelletier 2001). Only NMS plants must be crossed with male, pollen donating plants, to get a true hybrid, therefore cloning the mother plant to get an identical NMS population is necessary.

Leaf disc culture is a somatic tissue culture practice, as the explant origins from vegetative, non-sexual cells, meaning tissue cultured will be a genetic copy of the mother plants leaf. Callus growth is best initiated from the mid-rib of the first true leaf, figure 3. The callus, being undifferentiated cells, once grown sufficiently should then regenerate into shooting and rooting organogenesis (Dixon and Gonzales 1994; Owens and Eberts 1992).

We finalised a successful media for callus induction, and the maintenance of the callus in media. However the hormone concentrations were not perfect for shooting organogenesis, and therefore the callus did not differentiate into shoots during my time at CN Seeds. It is likely that the cytokinin concentrations are still too low for shooting (Trigiano and Gray 2011; Gaspar et al. 1996). The callus is being trialled in different medias, and hopefully we are close to narrowing down a successful media to trigger shooting, as the callus growth has been promising, figure 4.

The lack of results shows how time-consuming research of this nature can be. For instance it takes at least 2 weeks for callus to begin to respond to a new media, so it takes a lot of patience to trial different medias with ranging hormone concentrations and wait for growth responses. Though in context the actual time spent on the project each week is minimal, but it would be unrealistic to start this project and hope for immediate results without prior testing of the protocol. However once the right media is found this method can be used to multiply a mother plant quickly, likely within a month in the laboratory. These clones can then be used for hybrid seed production. Hybrid seed is sold at a commercial premium, as a true hybrid will have improved vigour, uniformity and resistance to stress.

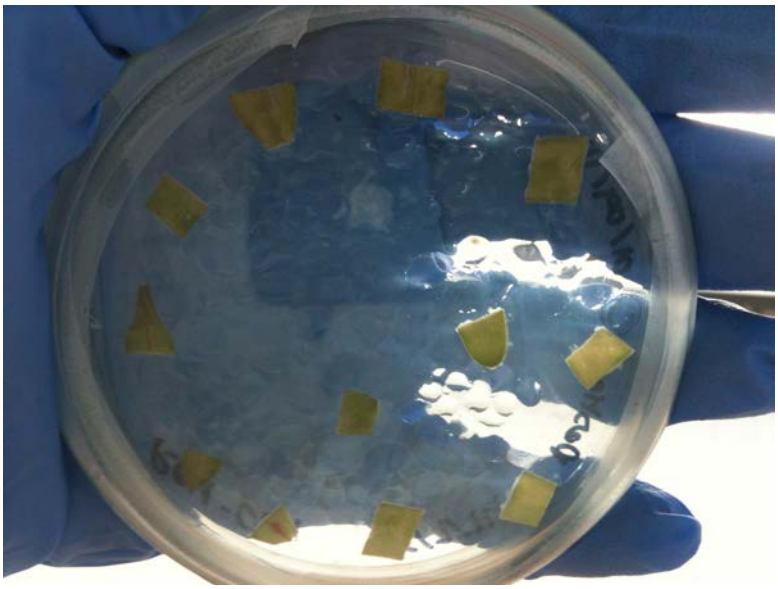


Figure 3. Leaf disc when first put in callus inducing media RCH-C1.

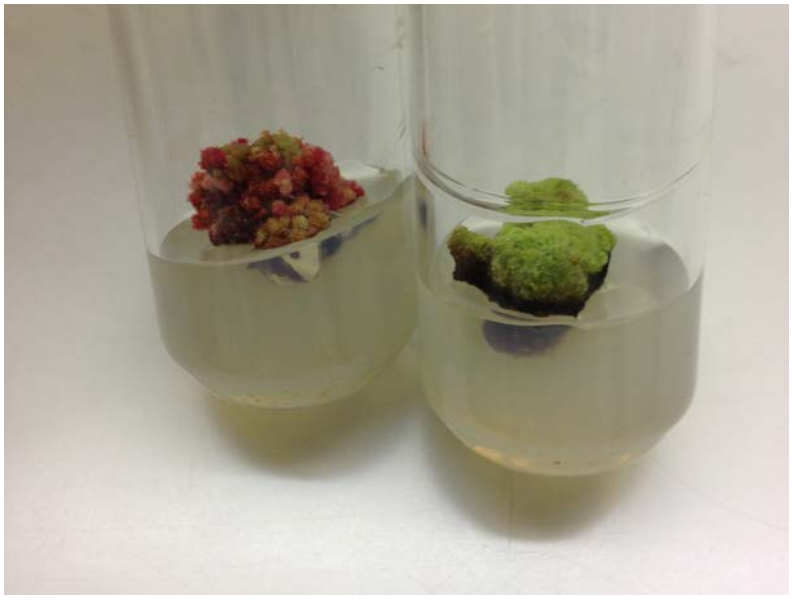


Figure 4. Promising callus growth, large calli with doming structures.

Meristem tissue culture

Meristem culture like leaf disc culture, is a method of multiplying identical plants with the same genetic makeup of the mother. Here the meristem tissue is cut from the mother plant (figure 5), be it found in the runners or the base of the shoots. This cut tissue once thoroughly sterilised will hopefully grow and proliferate many shoots (figure 6), which can be sub-cultured many times on agar, thus generating an indefinite number of plants from a single cutting (Martin 1985).

This is a popular method for multiplying plants such as strawberries which can have poor seed germination. Poor germination of seeds can be a result of inbred depression, which is the reduced biological fitness resulting from inbreeding (Keller and Waller 2002). Cloning is also desirable in strawberries to avoid trait segregation. Commercial strawberry cultivars are octoploid (Hummer et al. 2009) and therefore have a high level of trait segregation in successive populations, trait segregation follows Mendel's Laws of segregation and increases with ploidy level (Comai 2005). CN Seeds wanted to use meristem culture to multiply male and female strawberry plants for hybrid seed production, thus avoiding problems of trait segregation and inbred depression.

The main issue encountered with this technique was initiating the meristem tissue in the media. The explant itself often carried fungus internally, which would be terminal (figure 7). To combat infection I increased the number of sterilisation steps, whilst successively removing the outside tissue layers. In the end I was left with a very small piece of meristem tissue, I found there was a correlation between the smallest pieces of tissue and a reduced infection rate, indicating that fungus lay in-between tissue layers. If the tissue survived in the media without internal infection emerging we were able to grow and subculture the tissue easily before moving the shoots into rooting media and then cotton wool for hardening off. I found that the shoots once at a sufficient size would easily root after 6 weeks in a maintenance media, figure 8.

Overall this project was a success as we were able to tweak the protocol and propagate about 60 plants for trial hybrid seed production. The protocol if followed would take around three months to propagate a significant number of plants for hybrid seed production. This would overcome the issues of inbred depression and trait segregation.

Figure 5. Cut meristem tissue from a strawberry plant in SP-1 media.

Figure 6. Proliferating meristem which maybe sub-cultured many times to produce many plants.

Figure 7. Large meristem cuttings that have fungus infection. Fungus white and hairy.

Figure 8. Strawberry plantlets that have been removed from the final media; they have sufficient roots and shoots to be hardened of in cotton wool, then compost.

Embryo rescue

Embryo rescue was the last biotechnological technique tried during my placement and the one with the quickest success. In principle embryo rescue is performed on immature seed embryos, which would normally be aborted at some point in the seeds development. Embryo rescue is, as the name suggest, a way of salvaging the embryo from the mother plant and then raising and germinating the embryo on a specific nutrient medium, figure 9, thus bridging the gap between incompatible breeding species.

Related species can often successfully cross and set seed but then the seed will be aborted, often due to limited nutrients from the mother plant, or differing chromosome number of the parent plants (Ladizinsky 2012; Ndoutoumou et al. 2007). Therefore embryo rescue is a trusted tool for the plant breeder to cross interspecific lines and generate intermediate plants which can bridge the gap between related species, and therefore allows the transfer of traits between species. Here embryo rescue was used to obtain plants which were interspecific hybrids, and would have been unattainable through traditional breeding methods.

We found that the embryos of specific crosses, crosses of more closely related species were most successful. The protocol was successful with a range of different breeding lines. The most success occurring in embryos which were extracted from their pods at an older age, potentially due to their size and therefore robustness to being handled, bearing in mind the embryos are very delicate. Young embryos may lack specific nutrients, or have insufficient embryonic tissue differentiation, meaning further development is hindered (Ladizinsky 2012). Interestingly some embryos appeared to lay dormant, like seeds for a period of time, and therefore may need cold treatment to break dormancy (Bridgen 1994). Many embryos germinated in a specific embryo rescue media like normal seedlings, figure 10, the seedlings were grown in culture before being hardened off in the greenhouse.

Many embryo rescues were successful, and we were able to move them into the greenhouse. The plants grew well and flowered allowing crossings (figure 11), which means that the gap between species was successfully bridged using this technique. The plant breeders were able to use them in their current breeding program, demonstrating a very fast application of the technique.

Though some embryos have a higher success rate, often determined by the cross of the embryo, we were able to generate some plants from all crosses. Without embryo rescue these crosses would not have been viable. This technique will be used in the future, using the knowledge of the practise and the protocol, to generate more intermediate crosses. This has commercial advantages of being able to successfully add traits of interest to breeding lines, offering unquantifiable financial advantages in terms of breeding new commercial lines.

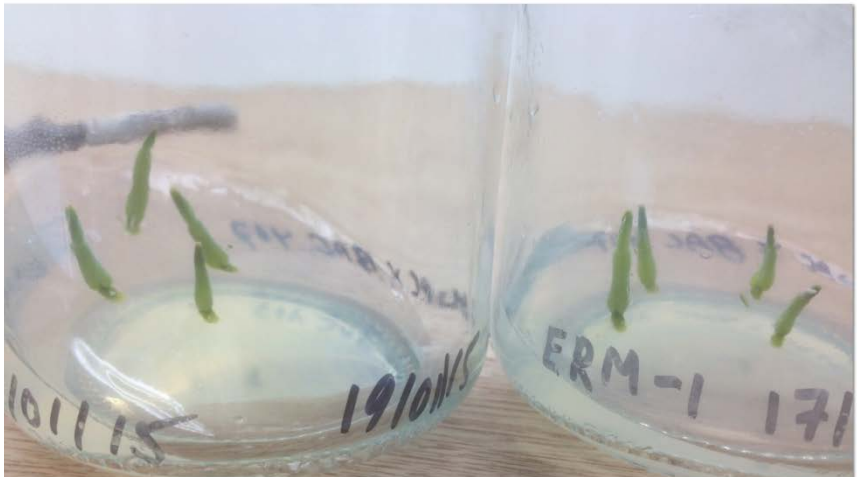


Figure 9. Pods cut from the mother plant and put in an embryo rescue medium, embryos will be excised from these pods and put onto fresh media after 1 week, and placed in the dark for germination.

Figure 10. Plantlet which germinated as a normal seedling from an excised embryo.

Figure 71. Flowering plant from an embryo rescue, which is ready to be crossed and used in a brassica breeding program.

Conclusion

Overall for a small laboratory many protocols were finalised for biotechnological techniques involving species which have had relatively little breeding improvement and therefore there is little published research available, meaning that applying these techniques to the specific species is generally unchartered territory. However with broad research of the techniques on related species, and generally a trial and error testing method we were able to develop protocols for specific species. The only technique trialled without complete success in the year was leaf disc culture, which has yet to have a shooting organogenesis 'breakthrough'. Though this project has some promising callus growth, so hopefully there will be success in the near future.

The protocols developed and techniques learnt will be of definite benefit to breeding programs at CN Seeds in the future, and in the case of embryo rescue the technique is already been used in current breeding programs. The ability to use biotechnology will offer a wider range of breeding possibilities along with many short cuts to traditional breeding programs, meaning that the time taken from the initial breeding stages to the commercialisation of a new variety is dramatically reduced. Anther culture reduces the number of selfing generations necessary to fix a trait, a time consuming task, as double haploid plants will not segregate traits in the successive generation. The use of leaf and meristem culture will allow for the rapid multiplication of identical populations that are difficult to produce through a seed cycles, either due to trait segregation or limiting factors such as inbred depression. Finally the possibility of transferring traits of interest between species, increasing the potential breeding traits, can be facilitated by the embryo rescue protocol successfully trialled. Therefore biotechnology will greatly help CN Seeds' plant breeders to create new and improved varieties, which will improve CN Seeds competitiveness in the ever demanding and evolving marketplace.

Appendix

Allele: a gene of 2 or more variants, which is found at a specific locus on a chromosome.

Auxin: plant growth hormone that stimulates cell division, in high concentrations it will stimulate adventurous root formation.

Cytokinin: plant growth hormone that stimulates cell division, in high concentrations it will stimulate adventurous shoot formation.

Dedifferentiation: when cells which were differentiated revert to undifferentiated, meristem cells.

Diploid: a cell with 2 chromosomes.

Explant: a cell, undifferentiated tissue or organ excised from a plant (or can be animal).

Gametophyte: a haploid multicellular form that in the alteration of generations will produce haploid gametes.

Gamete: a haploid cell such as egg or sperm.

Genetic recombination: when offspring have a different alleles (have different traits) to their parents following meiosis.

Hardening off: where a plantlet raised from tissue culture in a controlled climate is put in water, supported by cotton wool and then compost; meanwhile the temperature and humidity is slowly decreased to natural levels, which stimulates the plant to start using its stomata.

Inbred depression: the loss of vigour and fitness that is observed when genome-wide heterozygosity is decreased by inbreeding.

Interspecific hybrid: A cross of 2 different species.

Meristem: plant tissue that is undifferentiated.

Microspores: a spore from a land plant that develops into a male gametophyte.

Somatic: Non-sexual or vegetative stage.

Trait segregation: when alleles, expressed visually as the plants' growth traits, are recombinated after fertilisation leading to different traits in the next generation.

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