



Know-how for Horticulture™

**DNA finger printing
technology for purity
analysis of hybrid
seed in brassica crops**

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Henderson Seed Group P/L

Project Number: VG96030

VG96030

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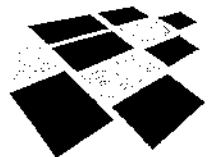
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This report summaries a partial fulfillment of a Ph. D. candidature by Phillip Crockett at Melbourne University. It highlights the use of DNA finger printing in the purity analysis of hybrid seed in *Brassica* crops. It compares the use of conventional grow out trial, isozyme electrophoresis and DNA finger printing in seed quality control in commercial *Brassica* hybrid seeds.

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Media Summary:

Many of the vegetable *Brassicas*, including cabbage, cauliflower and broccoli are grown from hybrid seed. An inherent obstacle for producing hybrid seeds of these crops is that they often produce self inbred (sib) seed. This seed when planted often produces less productive plants, and is therefore undesirable.

Seed purity analysis is a quality control requirement of hybrid seed producers. Common methods for determining sib levels in hybrid seed lots are grow out trials and isozyme analyses. This project was undertaken to establish whether the method of RAPD PCR (a form of DNA fingerprinting) was a useful alternative to these traditional methods.

The results found that DNA fingerprinting was a good alternative to currently used methods. It was especially useful in determining sib seed levels in hybrid seed produced from plants that were very closely related.

Some parental lines are very closely related and it can be difficult to identify sib seed levels using traditional methods. The results of this study revealed DNA fingerprinting to be a more powerful tool under these circumstances and would recommend its use in such cases.

Abstract:

Determination of genetic purity of F1-hybrid seeds is a quality control requirement in the production of hybrid *Brassica* seeds. Hybrid varieties of these vegetable crops have arisen from a limited germplasm base making discrimination of parental and hybrid lines laborious and troublesome. The use of RAPD PCR for evaluating seed purity in a commercial cabbage line (*Brassica oleracea* var. *capitata*) and a commercial broccoli line (*Brassica oleracea* var. *italica*) is demonstrated.

DNA from a F1-hybrids and their parental lines were subjected to RAPD PCR analysis with 20 random decamer primers. Primers producing suitable male and female specific markers were chosen for further analysis of hybrid seed lots. Concurrent studies on the same seed lots were also performed using the current methods of grow out trial and isozyme analysis.

The level of self-inbred seed detected for the cabbage line was comparable between all three methods. For the broccoli line isozyme analysis was unable to detect polymorphism between parental lines. Results from the grow out trial and RAPD analysis provided similar results.

The study clearly demonstrates that RAPD PCR is a useful alternative for seed purity analysis of vegetable *Brassica* hybrid seed lots. It was found to be particularly useful in the case of the broccoli line tested as the usual method of isozyme analysis was unable to differentiate between parental lines. This demonstrates RAPD PCR is genetically more powerful than isozyme analysis.

The use of RAPD PCR for seed purity analysis of vegetable *Brassicas*.

Introduction

Determination of genetic purity of F1-hybrid seeds is a quality control requirement in the production of hybrid *Brassica* vegetable seeds, to avoid unacceptable contamination with self-inbred (sib) seed. The grow-out trial is a commonly used method for seed purity analysis by commercial seed enterprises (Ballester and de Vicente, 1998). The trial consists of growing a representative number of hybrid seeds for phenotypic identification based on morphological characters that can vary according to environmental conditions. This type of trial is time consuming, space demanding, and usually performed off-season; therefore it often does not allow the unequivocal identification of the genotypes. Other methods, such as isozyme analysis and restriction fragment length polymorphism (RFLP), have also been used for the detection of contamination in hybrid seed lots (Arus et al., 1985; Livneh et al., 1990). Use of isozyme analysis is limited due to its inability to detect polymorphism among closely related genotypes. Though RFLP analyses reveal genetic variability, they are time consuming and labor intensive. Polymerase chain reaction (PCR)-based molecular marker techniques, such as randomly amplified polymorphic DNA (RAPD) (Williams et al., 1990) provide an alternative approach for evaluating genetic diversity (Asemota et al., 1996). These methods have proved useful in genetic mapping (Foisset et al., 1996), plant breeding, and genomic fingerprinting (Hu and Quiros, 1991; Lanham et al., 1995).

Brassica oleracea includes several important vegetable crops such as cabbage, broccoli, cauliflower, and kale. Of these *Brassica* vegetables, cabbage is the most widely grown vegetable. Its worldwide production was over 47 million mega tons in 1997 (Food and Agricultural Organization of the United Nations statistical database) making it an economically important crop. Commercial production of *Brassica* vegetables is usually achieved via hybrid seeds as hybrids are known to produce superior plants (Atlin et al., 1995). The production of hybrid seeds requires the control of self-inbreeding of parent plants. Self-inbred (sib) seeds produce plants that show less vigor, uniformity, and yields compared with the hybrids (Arcade et al., 1996) and are therefore undesirable contaminants. However, hybrid varieties of these vegetable crops have arisen from a limited germplasm base, making discrimination of parental and hybrid lines very laborious and troublesome. *Brassica* species generally display extensive polymorphism, however several studies have reported that of the vegetable brassicas commercial broccoli parental lines have arisen from a relatively narrow genetic base (Hu and Quiros, 1991; Gray, 1992).

Control of self-inbreeding in *Brassica* vegetables is predominantly dependent on the sporophytic self-incompatibility (SSI) character (Camargo et al., 1997). The SSI mechanism of these plants relies on complex dominant and co-dominant genetic interaction between alleles at the highly polymorphic S-locus of the male sporophyte and the cells of the stigmatic papillae. These complex genetic interactions result in production of variable amounts of sib seeds in hybrid crosses. In addition,

environmental conditions are also known to influence the efficiency of SSI (Bell, 1995). Therefore, every hybrid seed harvest needs to be tested for the presence of inbred seeds prior to the release on the market.

The need for seed purity testing

Numerous reports and observations have shown that crosses between genetically different parents often result in superior progeny, a phenomenon known as heterosis (Arcade et al., 1996). Hybrid plants show greater vigor, uniformity and yield (Nijenhuis 1971; Arus et al., 1982; Atlin et al., 1995). Arcade et al., (1996) reported a study on larch species (*Larix decidua* and *Larix karmpferi*) which utilized molecular markers to determine the genetic relatedness of parental lines. Comparison of the resultant hybrids showed a positive correlation between genetic distance of the parents and growth performance of the hybrids. Hybrid seed produces superior plants therefore many crop species are now planted with hybrid seed. These include maize, sunflower, sorghum, sugarbeet, cotton and many vegetable species. Hybrid seed production in the vegetable *Brassica*'s occurs by the crossing of two inbred parents, stable for agronomically important traits. A problem that must be overcome in hybrid seed production is self-inbreeding of parental plants. Self-inbreeding occurs when a plant is fertilized by its own pollen, resulting in non-hybrid, self inbred (sib) plants suffering from inbreeding depression. These sib plants show less vigor, uniformity and yield, with respect to the hybrid (Arcade et al., 1996), and therefore are undesirable contaminants in the hybrid seed lot.

Overcoming self-inbreeding

There are several breeding strategies for overcoming self-inbreeding. Often these systems are not 100% efficient, therefore it is necessary that hybrid seed lots be tested, as a means of quality control, for seed purity before they are placed on the market (Wills et al., 1979; Arus et al., 1982; Rom et al., 1995).

Male Sterility

Many breeding systems now incorporate cytoplasmic male sterility (CMS) which renders one parental line male sterile, hence it is used as the female parent. For reviews refer to Sahwney and Shukla (1994) and Lasa and Bosemark (1994). Male sterility is employed in several crop species including millet, rice, sorghum, and sunflower. *Brassica napus* (oilseed rape) is also a crop for which most hybrid cultivars have been developed using cytoplasmically inherited male sterility (Atlin et al., 1995; Liu et al., 1996). More recently the transfer of CMS into the vegetable crops of *Brassica oleracea* has been reported (Cardi and Earle, 1997; Sigareva and Earle, 1997). The introduction of CMS can be achieved by the traditional crossing of commercial varieties to naturally occurring CMS species (Kirti et al., 1995), or by other methods such as protoplast fusion (Cardi and Earle, 1997). Whilst the CMS system is slowly being incorporated into the *B. oleracea* vegetable crops, much of this work is still in developmental stages. The majority of hybrid seed production in these

vegetables is still dependent on the self-incompatibility (SI) system for controlling self-inbred seed, hence purity testing is still required.

Self-incompatibility

Self-incompatibility (SI) is the inability of plants to produce seed when they have been self-pollinated (Matton et al. 1994). It has been assumed that the first angiosperms were self-compatible and that SI is a derived condition which promotes outbreeding (Bell, 1995; Elleman and Dickinson, 1994). SI is an example of cell to cell recognition in plants, with the cells of the stigmatic papillae (cells which receive pollen) being able to determine self and non-self, arresting the growth of self pollen, hence maintaining variability (Matton et al., 1994).

There are two types of SI; sporophytic self-incompatibility (SSI) which occurs in the *Brassicaceae* and *Asteraceae* (daisy) families and the more common gametophytic self-incompatibility (GSI) which occurs in the *Solanaceae* (Poppy) family and the *Onagraceae* (Charlesworth, 1995).

Sporophytic self-incompatibility (SSI)

Sporophytic self-incompatibility is less common than GSI but is the mechanism applicable to the *Brassicaceae*. SSI has been widely studied in *B. oleracea* with many genotypes displaying SI. The system has been utilized to produce hybrids of broccoli, cabbage, cauliflower and kale (Camargo et al., 1995). The SI character is determined by a single multi-allelic locus, termed the S-locus. The complement of allelic genes at the S-locus is described as the S-haplotype. Approximately 60 S-haplotypes have been described for *Brassica* (Matton et al., 1994; Bell et al., 1995; Camargo et al., 1997; Ruffio-Chable et al., 1997), making this locus one of the most polymorphic known. Rejection of self pollen is controlled by the genotype of the stigma and that of the pollen parent. As the division of the generative nucleus into sperm cells has already occurred when pollen grains are shed, the phenotype of the germinating pollen is determined by the parent sporophyte, hence 'sporophytic' self-incompatibility. In SSI both the pollen and the stigma present 2 alleles, if any of these match then rejection can occur (Matton, 1994; Bell, 1995). As mentioned the S-locus is highly polymorphic which results in complex dominant and co-dominant interaction which affect the outcome of crosses. Different SI alleles show different levels of efficiency and this efficiency has also been shown to be prone to environmental conditions (Wills et al., 1979; Arus et al., 1982). In an incompatible reaction pollen fails to hydrate, or hydrates very slowly. If germination does occur the stigmatic papillae resist penetration by the formation of a callosic pad beneath the cuticle (Herrero and Dickinson, 1981; Ellemen and Dickinson, 1994).

Three genes have now been identified within the S-locus, the first being the S-locus glycoprotein (*SLG*) gene, which encodes an extracellular glycoprotein. The second gene, S-locus receptor kinase encodes a membrane associated protein that reportedly phosphorylates serine and threonine residues (Matton et al., 1994). Boyes and Nasrallah (1995) reported a third gene, S-locus anther (*SLA*) gene. All of these genes have been shown to be expressed in reproductive structures of the flower, with SLG

being abundant in stigmatic papillae (Gaude et al., 1995). There is some doubt however of the role of SLG in the SI reaction as it has been reported that some SI haplotypes, such as S2 in *B. oleracea*, express very little SLG glycoprotein yet display the SI phenotype (Gaude et al., 1995). Camargo et al., (1997) located the S-locus on a RFLP and RAPD marker generated linkage map and found *SLG* and *SLK* to be tightly linked in *B. oleracea*. These genes were also found to be very similar with 80% amino acid homology.

S-locus alleles have been divided into two classes. Class I alleles are high in activity (dominant) and exhibit a strong SI phenotype. Class II are of low activity (recessive). This classification has recently been questioned as a class I haplotype was found to be associated with full self-compatible phenotypes (Ruffio-Chable et al., 1997).

Gametic self-incompatibility (GSI)

GSI is more common than SSI and has been well characterized in the *Solenaceae* (Tobacco, petunia, potato and tomato) (Matton et al., 1994). As with SSI, GSI is controlled by a single multi-allelic S-locus. Rejection of self pollen however occurs as a result of interaction of the single S allele present in the haploid pollen grain (gamete) and the two S alleles of the diploid stigma. Similarly to the SSI system *SLG* and *SLK* genes have been identified, the SLG glycoproteins commonly exhibiting RNase activity (McClure et al., 1989). These glycoproteins have been named S-RNases. The common perception is that in an incompatible reaction the S-RNases enter the pollen tube where they degrade RNA. The degradation of this RNA is proposed to arrest the growth of the pollen tubes (Matton et al., 1994). However this theory has been challenged by Bell (1995) who examined previously published electron micrographs and found them to show no areas of digestion or loss of ribosomes. A study by Foote et al., (1994) showed that in poppy, which displays 100% GSI phenotype, that the SLG glycoproteins display no RNase activity. This finding raises questions of the role of SLG in GSI.

Testing seed purity

Extensive research has been focussed on characterizing SI mechanisms, however much is still not understood. What is known however is that the system of self-incompatibility is a complex one, and the variation in efficiency of different SI alleles makes this system difficult to work with for breeding, and hybrid seed production. The desirable system appears to be male sterility. The majority of hybrid seed production for the vegetable *brassica*'s is still dependant on the SI system, therefore it is still a requirement that seed purity testing be performed before seed is placed on the market. There have been two primary methods for seed purity testing utilized by hybrid seed companies, grow out trials, and isozyme analysis. The relatively more recent DNA based methods of restriction fragment length polymorphisms (RFLP) and the random amplified polymorphic DNA (RAPD) PCR analysis have been explored in some plant families for their usefulness in seed purity testing, and varietal studies.

Grow out trial

A grow out trial is simply the planting out of a representative number of seeds (commonly 300) and allowing the plants to grow in the field. Depending on the plant under trial this process may take 12 weeks or more to complete. Three readings are usually taken during this period and self-inbred plants are identified phenotypically. This method whilst being relatively simple is time consuming, labor intensive and requires large areas of land.

Isozyme analysis

Smithies, (1955) first advanced the technique of electrophoresis as a tool for geneticists. Electrophoretic analysis of proteins and enzymes was first used for studying phylogenetic relationships between species of the genus *Brassica* in the late 1960's (Vaughan and Waite, 1966; Vaughan and Denford, 1968; and Yadava et al., 1979). The first use of isozyme analysis for intra specific variation in *B. oleracea* was reported by Nijenhuis (1971) who studied banding patterns for acid phosphatase. In this report he also proposed the use of isozyme analysis for seed purity testing. Since this report further research such as that of Woods and Thurman (1976), Wills et al., (1979), Wills and Wiseman, (1980), Arus and Orton (1983) and Chevre et al., (1995) has identified many useful loci for intra-specific variation and purity studies in *Brassica*. Table 6.1 lists enzyme loci that have been characterized in *B. oleracea* and the relevant references.

As outlined above, isozyme analysis is a method that has been utilized for nearly 30 years in *Brassica*, and whilst there have been some slight changes and advancement of methods, it is still predominantly unchanged. Several forms of electrophoresis can be used to analyze isozymes including polyacrylamide gel electrophoresis, sodium acetate gel electrophoresis, agarose gel electrophoresis, and starch gel electrophoresis. Whilst the buffer and gel systems used may be different the basic banding patterns remain consistent. For reviews of methods used see Shields et al., (1982), Murphy et al., (1990). Starch gel electrophoresis is the most commonly used system for seed purity testing, utilized by many seed companies. It is relatively cheap, simplistic and the gels can be sliced into several layers after running therefore allowing for the analysis of several loci from one gel. Isozyme analysis has also proved useful for the determination of genetic relationships. Although much of this work is now being supplemented or completely achieved using DNA based methods much research still utilizes analysis of isozymes. For example Simonsen and Heneen (1995) utilized ten different isozymes to study genetic variation in *B. oleracea* whilst Sekhon and Gupta, (1995) utilized isozymes for determining genetic differences between parental lines and predicting heterosis in the F1 generation of *Brassica* species.

Table 1: Isozyme loci characterized in *Brassica oleracea*

Isozyme	References
Acid Phosphatase (APS)	Nijenhuis, 1971; Woods and Thurman, 1976; Wiseman and Wills, 1976; Wills et al., 1979; Arus and Orton, 1983; Arus and Shields, 1983; Chevre et al., 1995.
Alpha-amylase	Wills et al., 1979
Carboxylesterase	Wills et al., 1979
Beta-galactosidase	Wills et al., 1979
Beta-glucosidase	Wills et al., 1979
Leucine aminopeptidase	Shaw and Koen, 1970; Wills et al., 1979; Arus et al., 1982; Arus and Orton, 1983; Arus and Shields, 1983; Chevre et al., 1995.
Peroxidase	Wills et al., 1979
Phosphoglucosmutase	Tanksley, 1979; Arus et al., 1982; Coulthard and Denford, 1982; Arus and Orton 1983; Chevre et al., 1995.
Phosphoglucose isomerase	Tanksley, 1980; Arus et al., 1982; Arus and Orton, 1983.
Catalase	Scandalios, 1969; Yadava et al., 1979.
Esterase	Reddy and Garber, 1971; Yadava et al., 1979.
Alcohol dehydrogenase	Scandalios, 1969; Yadava et al., 1979.
Glutamate-oxalacetate transaminase	Rick et al., 1977; Arus and Shields, 1983.
6-phosphoglucose dehydrogenase	Quiros et al., 1987; Chevre et al., 1995.
Triosephosphate isomerase	Quiros et al., 1987; Dias et al., 1994; Chevre et al., 1995.
Aspartate aminotransferase	Dias et al., 1994.
Glutathione reductase	Dias et al., 1994.
Superoxide dismutase	Dias et al., 1994.
Isocitrate dehydrogenase	Dias et al., 1994.
Fructose biphosphatase	Dias et al., 1994.
Aconitase	Arus, 1989; Chevre et al., 1995.

Whilst isozyme analysis is an old technique it is still the preferred method for purity testing by seed companies, due to its relative low cost and simplicity. Since the introduction of more powerful DNA based techniques in 1982 several methods have been assessed for their usefulness in genetic studies, including seed purity testing.

DNA based seed purity testing

The use of isozyme analysis is not always possible for seed purity testing of closely related genotypes due to lack of polymorphism (Hashizume et al., 1993). For this reason more powerful molecular techniques are now being utilized.

Restriction fragment length polymorphism (RFLP) analysis was the first DNA profiling technique to be utilized for the study of plant variation. The technique involves the extraction and purification of genomic DNA, followed by digestion with restriction endonucleases, which cut the genomic DNA into fragments. These fragments are then separated by agarose gel electrophoresis, transferred to a membrane and specific fragments are identified using specific, labeled, DNA probes. Maize, wheat and soybean have been extensively studied using RFLP's and as a result a large number of probes are available, however this is not so for all species (Morell et al., 1995). RFLP's have been used successfully in many studies of *B. oleracea*. Examples include the mapping of quantitative trait loci controlling flowering time (Camargo and Osborn, 1996) and clubroot resistance markers (Figdore et al., 1993). RFLP's have also been widely used for studying genetic relationships between and within species (Tivang et al., 1994; Dias, 1994). Whilst RFLP analysis is a powerful genetic tool it is relatively time consuming when compared to isozyme electrophoretic analysis. The technique is more costly, requires a higher level of skill and working with radioactive material, therefore little research into seed purity testing using this method has been reported.

Williams et al., (1990), reported a technique that has been utilized more recently for varietal identification. This technique, random amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR), requires the use of short DNA primers (commonly decamers) to perform PCR on genomic DNA. This results in the amplification of regions for which the primers bind closely enough on opposite strands of the DNA. Variation in DNA sequence (polymorphism) can result in different banding patterns which are assessed by agarose gel electrophoresis. RAPD markers are inherited in Mendelian fashion as dominant markers (Williams et al., 1990). RAPD PCR, whilst having similar genetic resolution to RFLP's (dos Santos et al., 1994; Hallden et al., 1994) has several advantages over RFLP which includes only small amounts of DNA being required, no knowledge of the sequence is required, no radioactive work is performed and the analysis is simpler. This method is also less labor intensive and faster. The applications for RAPD PCR are very similar to that of RFLP's. Whilst the consistency of the technique has been questioned many authors have recorded consistent results. The use of RAPD PCR for purity testing has been reported in chicory (Bellamy et al., 1996), *B. napus* (Marshall et al., 1994), tomato (Rom et al., 1995), and watermelon (Hashizume et al., 1993). Hu and Quiros, (1991) demonstrated the use of RAPD PCR in comparison of broccoli and cauliflower cultivars.

Aims

The objectives of this study were to establish whether RAPD markers could be used for hybrid seed purity testing of commercial cabbage and broccoli cultivars and to compare results from RAPD analysis with the commonly used methods of grow-out trial and isozyme analysis.

Materials and Methods

Plant material

Four seed lots of cabbage, *Brassica oleracea* var. *capitata*, and four seed lots of broccoli, *Brassica oleracea* var. *italica* were provided by the Henderson Seed Group Pty, Ltd., Templestowe, Victoria, Australia. Male parent, female parent, and the F1-hybrid seeds harvested from the male line (the male harvest), and the female line (the female harvest), were provided for both a commercial cabbage and broccoli line. The seeds were stored, desiccated at 4°C until used.

Genomic DNA isolation

Two types of plant material; seed and three week old seedlings were compared for genomic DNA isolation. Seedlings were grown from seeds in a commercial potting mix under glasshouse conditions. Three DNA isolation methods were tested in this study. Genomic DNA from leaves of seedlings was isolated following a scaled down protocol of Dellaporta et al., (1983). A protocol based on the method of McDonald et al., (1994) was used for genomic DNA extraction from individual seeds. The modifications of the McDonald et al., (1994) protocol included RNase treatment of isolated DNA followed by phenol chloroform extraction according to Sambrook et al., (1989). The Easy-DNA™ (Invitrogen) kit was also used to extract DNA from seedlings. The quality of DNA was assessed by electrophoresis on a 0.7% agarose gels containing ethidium bromide (0.5 ug/ml) in 1X TBE (89mM Tris-HCL, pH 8.3, 89 mM boric acid, 5 mM EDTA). DNA was quantified using a DyNA Quant™ fluorometer (Hoefer Pharmacia Biotech. Inc.). The final concentration of the DNA was adjusted to 12.5 ng/ul.

RAPD amplification

RAPD primers (OPA and OPB series) obtained from Operon Technologies, Inc. (Alameda, Calif.) were tested.

PCR was performed in a volume of 20 ul containing 10 mM Tris-HCl (pH8.3), 50 mM KCl, 2 mM MgCl₂, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 25 uM primer, 1.5 U Taq DNA polymerase (Gibco-BRL), and 25 ng of template DNA. A Perkin-Elmer GeneAMP PCR system 2400 thermocycler was used. The PCR program used was the same as described by Swoboda and Bhalla (1997); initial denaturation step at 94°C for 4 min, followed by 45 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 2 min, and extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min. The amplification products were separated by electrophoresis on 1.5% agarose gels, stained with ethidium bromide, and photographed under UV light (302 nm) using polaroid 667 film.

Isozyme analysis

Crude extracts were prepared by crushing individual seeds in 40 μ l of extraction buffer (0.1 M Tris, 1.0% (w/v) reduced glutathione, pH 8.5) in microtitre plates. Extracts were then taken up in Whatmann paper wicks and loaded onto 11% starch (Sigma) gels. Gels were run at 275 volts at 4 °C. Two buffer systems; tris-citrate/borate (Shields et al., 1983) and tris-citrate II (Murphy et al., 1990) were evaluated. Three enzymes reported to be highly polymorphic in *Brassica* species (Chen et al., 1989) were examined: Phosphoglucosmutase (PGM), phosphoglucose isomerase (PGI), and leucine amino peptidase (LAP). Enzyme staining was performed following the protocol of Arus and Shields, (1983).

Grow out trials

Plants were grown from seeds for 8 weeks under glasshouse conditions and then transplanted into the field for subsequent growth and phenotypic evaluation. 275 plants from the cabbage male harvest and 266 plants from the female harvest were assessed. 171 plants from the broccoli male harvest and 192 from the female harvest were assessed. Plants were evaluated for the presence of sib contaminants at 8, 12, and 14 weeks after transplanting.

Results

Genomic DNA isolation

The suitability of single seed and leaves of seedlings for genomic DNA isolation a RAPD analysis was compared. DNA isolation from leaf samples requires seed germination and plant growth, which makes the process time consuming and labor intensive. Reduction in time and labor for DNA isolation is required for the practical application of RAPD analysis in seed purity testing. DNA extraction from seeds offers an alternative for seed purity testing. Our results indicate that the quality and quantity of DNA extracted from single ungerminated seeds is sufficient for RAPD analysis. The modified method of McDonald et al., (1994) was found to be simple and fast as compared to genomic DNA isolation from young leaves of seedlings using the Dellaporta et al, (1983) method. Using the Easy-DNA™ extraction kit from Invitrogen was a faster procedure than the Dellaporta method, and produced DNA of usable quality however the DNA extraction kits are costly. Further, the DNA isolated from single ungerminated seed was found to be suitable for seed purity analysis with the selected RAPD markers. It is worth mentioning that DNA extracted from single seed produced less bands than seedling DNA, and some of the less prominent (weakly stained bands) markers were not reproducible. This may be due to the presence of impurities in the DNA preparation as seeds are known to contain significant amounts of polysaccharides, phenolics, and lipids. Nevertheless the selected RAPD markers were found to be highly reproducible using DNA from both seed and seedling.

RAPD analysis

In many crops the F1-hybrid seeds are only collected from one parent, however, for the cabbage cultivar tested in this study we used hybrid seeds from both the male and female parents, because commercial F1-hybrid seeds of this cabbage are sold as a blend from male and female harvest.

A total of 36 primers were screened for analysis of the cabbage line, and primers that produced strongly stained polymorphic markers were further tested for reproducibility by repeating reactions at least three times. Figure 6.1 shows an example of 12 primers. After testing reproducibility of markers, 20 plants of each parental line were tested to validate the homogeneity. Two primers, OPA-07 (GAAACGGGTG) and OPB-08 (GTCCACACGG), were subsequently chosen for seed purity analysis (Figure 6.2). Of the 36 primers tested, 30 produced polymorphic bands. A total of 241 products, ranging from approximately 0.5 kb to 5 kb, were observed with 54 (22%) being polymorphic. Primer OPB-08 produced a male parent specific marker and was chosen to screen F1 seeds from the female harvest. Use of the OPA-07 primer resulting in the amplification of a female parent specific band and it was utilized to screen the hybrid seeds from the male harvest (Figure 6.3). RAPD amplification of DNA extracted from individual seeds from the female harvest revealed that 12 of 132 (9%) seeds tested were self-inbred. RAPD analysis of hybrid seeds from the male harvest revealed 22 of the 126 (17%) seeds tested were sibs.

F1 hybrid seeds are usually collected from one parent (female parent). For the analysis of the broccoli line we tested hybrid seeds from both the male and female parents, as both parental lines have been used as female lines in previous crosses for hybrid seed production. A total of 20 primers were tested, with 15 primers showing some polymorphism between the two parental lines. Two primers (OPB-04, OPB-12) were chosen for further studies. These primers were tested for homozygosity of the selected markers in the parental lines by analyzing the DNA of 20 individual male of female parental plants. OPB-12 produced a male specific band and was used to screen the female harvest. OPB-04 produced a female specific band and was used to screen the male harvest (Figure 6.4). RAPD PCR analysis of 100 progeny from both of the F1 harvests revealed 45 and 14 seeds of the female and male harvest to be sibs respectively.

Isozyme analysis

Isozyme analysis revealed no polymorphism between the broccoli parental lines. A total of 50 seeds from each cabbage parental line were tested to ensure homozygosity, and reproducibility of polymorphic markers. Once polymorphism was established, 150 seeds of both the male and female F1-hybrid seed harvest were tested for the presence of sib seed contamination. Use of phosphoglucosyltransferase (PGM) and phosphoglucose isomerase (PGI) produced polymorphic markers between the cabbage male and female parental lines, but leucine amino peptidase failed to detect any polymorphism. The resolution of the bands was considerably better when PGM was used. One hundred and fifty seeds from both the male and female harvest were subsequently screened for seed purity using PGM isozyme markers (Figure 6.5). Results from these experiments revealed that 12 (8%) seeds from the female harvest and 25 (17%) seeds from the male harvest were sibs.

Grow-out trial

Plant characters including height, plant shape, and leaf shape were considered when scoring the level of sibs in the hybrid seeds. Over the three observations, from 8 to 14 weeks after transplanting, the number of sibs scored increased as phenotypic characters were more easily distinguished. For the cabbage female F1 harvest 18 of 266 (7%) of plants were scored as sibs and 38 of 275 (14%) of the male harvest. For the broccoli female harvest 82 of 192 plants (43%) and 21 of 171 plants (12%) of the male harvest were scored as sibs. Table 6.2 summarizes the results of all three methods for the cabbage line tested and Table 6.3 summarizes the results for the broccoli line tested.

Figure 1: Analysis of male parent and female parent DNA of a hybrid cabbage cultivar with 12 RAPD primers. The primer used is indicated above the lanes.

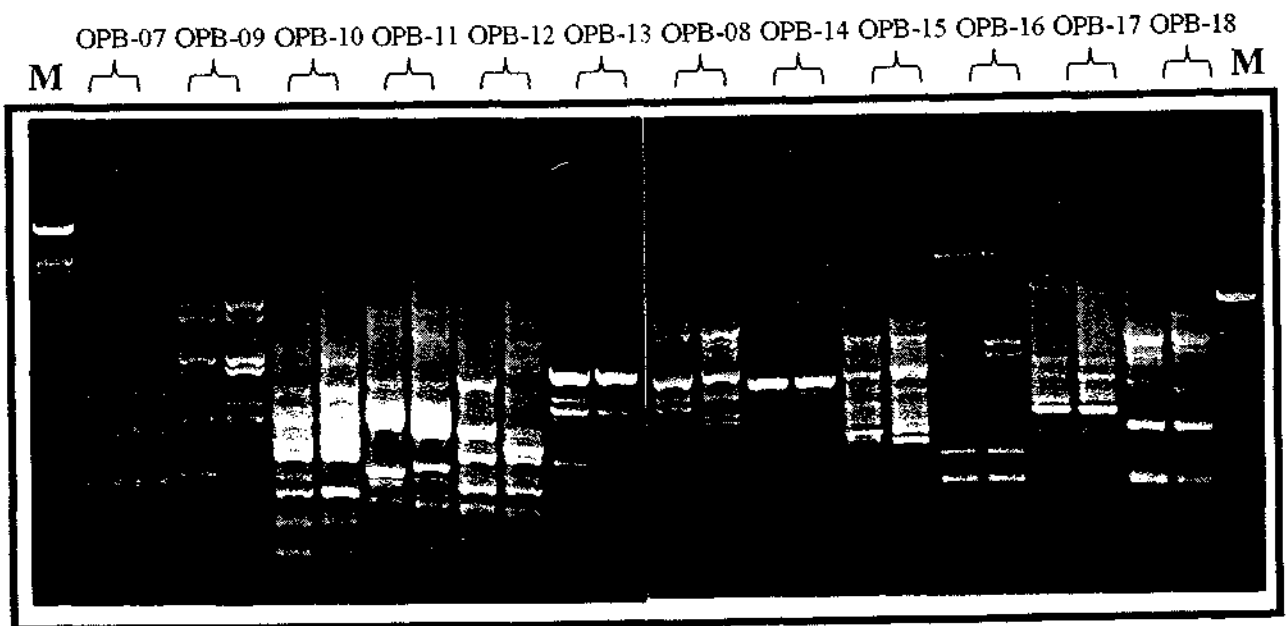


Figure 2: RAPD profiles of female parent (FP), male parent (MP) and their F1 hybrid (F1) with primer OPA-07 and OPB-08. Arrows indicate the female parent and male parent specific markers generated using these primers. M, molecular size markers.

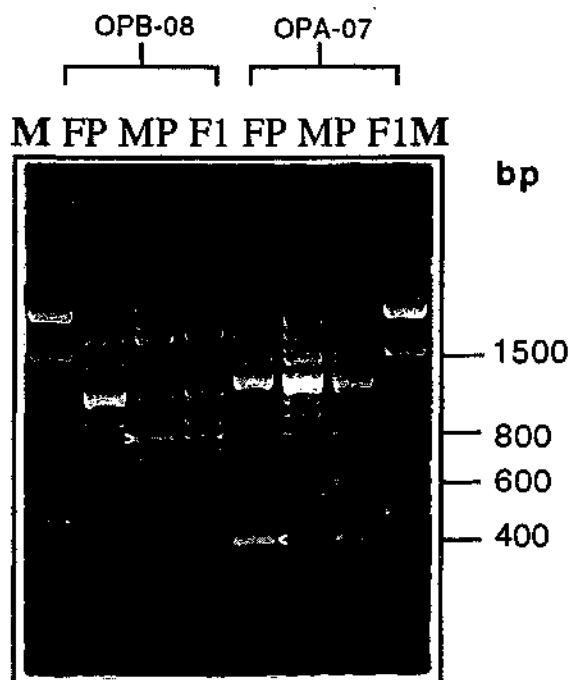
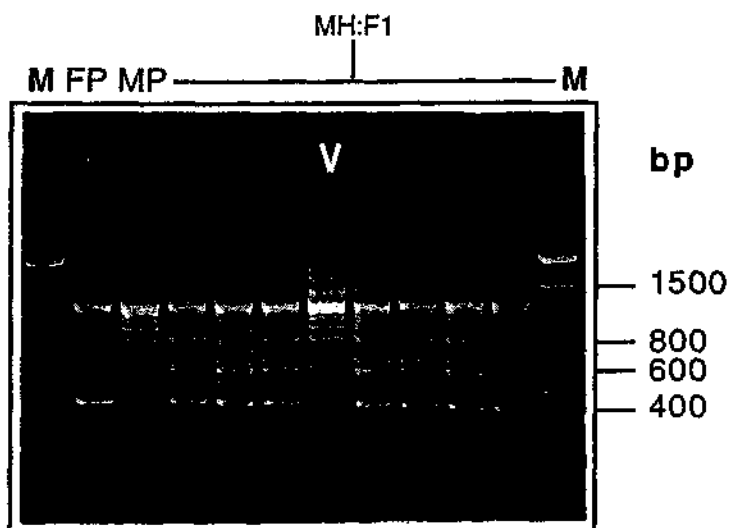


Figure 3:

Seed purity analysis using RAPD profiles generated with OPA-07; Female parent (FP), male parent (MP) and the F1 hybrid (F1) harvested from the male plant (MH:F1). The arrow indicates the self-inbred seed. M, molecular size marker.



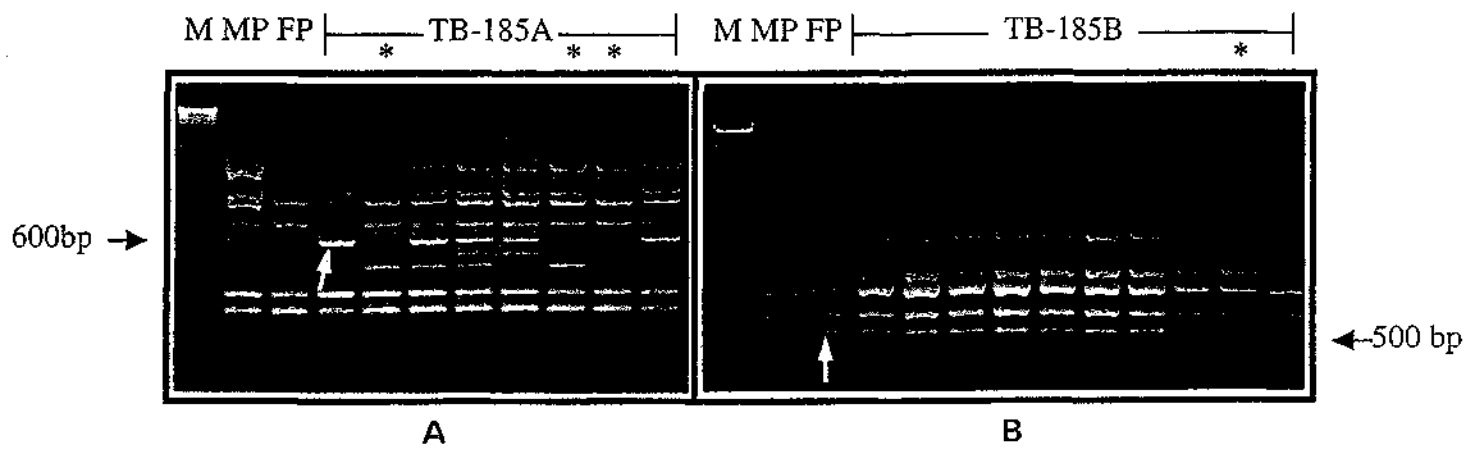


Figure 4: RAPD profiles of broccoli F1 hybrid seeds. M, molecular weight marker. RAPD markers used for purity analysis are marked with arrows, and lanes containing self-inbred samples are marked with an asterisk. A) shows analysis of male parent (MP), female parent (FP) and the F1 hybrids from the female harvest (TB-185A) with primer OPB-12. B) shows the analysis of MP, FP and F1 hybrids from the male harvest (TB-185B) with primer OPB-04.

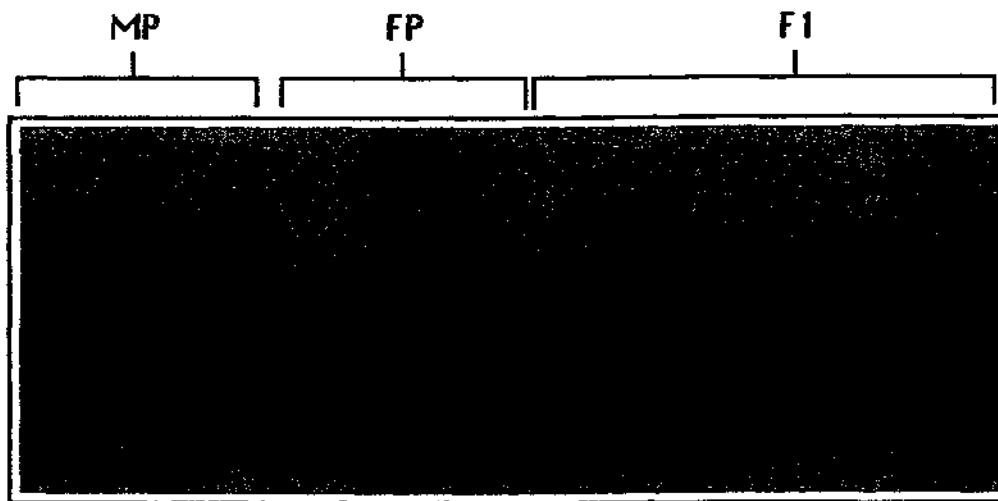


Figure 5: Phosphoglucomutase (PGM) isozyme polymorphism displayed on starch gel. MP, male parent; FP, female parent; F1, hybrid.

Table 2: Comparison of the number of sibs detected in a commercial cabbage cultivar using three different methods

Method Used	Total seeds tested	Number of Sibs	% Sibs
Grow out trial			
Female Harvest	266	18	7
Male Harvest	275	38	15
Isozyme analysis			
Female Harvest	132	12	9
Male Harvest	126	22	18
RAPD analysis			
Female Harvest	150	12	8
Male Harvest	150	25	17

Table 3: Genetic purity of hybrid broccoli seeds obtained using RAPD PCR analysis and grow out trial

Method Used	Total Seeds Tested	Number of Sibs	% Sibs
RAPD analysis			
Female Harvest	100	45	45
Male Harvest	100	14	14
Grow out trial			
Female Harvest	192	82	43
Male Harvest	171	21	12

Discussion

Prerequisites for the use of RAPD's as a routine method for seed purity testing at the commercial scale are simplicity, efficiency, reliability and minimal cost. For this reason, two standard DNA extraction methods from seedlings and a relatively faster DNA isolation method directly from seeds, were compared. Both tissue types and the three DNA extraction methods tested provided reproducible results for the selected RAPD markers. Less prominent bands amplified from seed DNA were found to be less reproducible as observed previously by Hallden et al., (1996) indicating that more intensely stained bands should be chosen as RAPD markers for seed purity testing. Several reports in the literature question the consistency, validity and the ability to reproduce RAPD markers (Thormann et al., 1994; Hallden et al., 1996). In this study no difficulty was encountered in reproducing the selected markers. Some bands, especially those smaller than 400 base pairs and larger than 1500 base pairs were difficult to reproduce. Unlike phylogenetic studies where several bands produced by a RAPD primer are analyzed (Jain et al., 1999) purity analysis requires the analysis of only one distinct marker.

The results shown in this chapter show that RAPD analysis can be used as a method for seed purity testing of commercial *Brassica* vegetables. No significant difference was observed between the levels of sibs detected using any of the methods tested. The study also revealed significant polymorphism between the parental lines studied using RAPD PCR. Isozyme analysis was found to be useful for detecting polymorphism in the cabbage cultivar tested, but not the broccoli lines. It has been reported previously that broccoli parental lines of commercial seed companies are closely related (Hu and Quiros, 1991). The reasons given that the original parental lines displaying desirable agronomic traits arose from limited germplasm base and were preferentially used for breeding of new parental lines for hybrid seed production. With time it is therefore possible that parental lines within one company will become more closely related.

Isozyme patterns can differ dependant on the environmental conditions and the plant tissue used. Moreover, use of isozyme analysis are known to be limited as indicated by their failure to detect polymorphism in more closely related parental lines of watermelon, tomato, and broccoli. In contrast, the application of RAPD technique has been shown to be useful in differentiating closely related parental lines of these plants as demonstrated here and in previous work (Hashizume et al., 1993).

Hybrid seed production in vegetable *brassica's* is dependent on sporophytic self-incompatibility to prevent self-pollination. The self-incompatibility barrier is often breached by environmental factors such as high temperature and humidity leading to the formation of selfed seed, hence contamination in the hybrid seed harvest. Commonly used grow-out trials based on morphological identification of parental and hybrid genotypes are labor intensive and time consuming. The results provided here show that RAPDs can be successfully employed for evaluation of sib contamination in hybrid seeds of commercial cabbage and broccoli lines and that there is a significant advantage in using RAPD markers over other commonly used methods. In addition, single seeds can be used for the DNA isolation making the use of RAPD markers efficient, fast and less labor intensive for seed purity testing. As PCR

techniques become more and more simplified as well as DNA extraction techniques it is likely that RAPD PCR or similar methods may well supersede the current methods of purity analysis. Isozyme analysis is still probably the preferred method because of its simplicity and comparatively less setup cost. However RAPD PCR is a useful alternative to isozyme analysis when polymorphism can not be detected.

Recommendations

The results of the research clearly demonstrate RAPD PCR as a useful tool for analysis of seed purity in vegetable *brassicas*. This method is most useful in purity analysis of lines that are too closely related to be separated using isozyme analysis, a commonly used method.

Isozyme analysis still remains the preferred method due to its simplicity and relatively lower setup costs. More research is required into much simplified DNA extraction methods and high throughput PCR before a method such as RAPD PCR with supersede isozyme analysis.

The findings of this research would recommend the use of RAPD PCR for the detection of sib purity levels in lines unable to be differentiated using isozyme analysis.

Technology Transfer

The use of RAPD markers for evaluating seed purity in commercial F1 Brassica hybrids has been demonstrated. RAPD PCR was found to be a useful tool for analysis of hybrids with parent lines that cannot be separated using isozyme electrophoresis. The RAPD PCR method was used by Henderson Seed Pty. Ltd. to test the purity in one of its commercial broccoli variety.

The methods were published in International Scientific Journals.

1. RAPD analysis of seed purity in a commercial hybrid cabbage (*Brassica oleracea* var. *capitata*) cultivar.

Phillip A. Crockett, Prem L. Bhalla, C.K. Lee, and Mohan B. Singh.

2. Genetic purity analysis of hybrid broccoli (*Brassica oleracea italica*) seed using RAPD PCR.

Australian Journal of Agri Res. (In Press).

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