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# Molecular detection for adventitious presence of fertile seeds of rapeseed in Ogura-type sterile seed lots

Seyed Hossein Jamali, Maryam Najafian, Saman Sheidaei, and Leila Sadeghi

Seed & Plant Certification & Registration Institute, Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran

***Corresponding authors**: jamaly.h@gmail.com

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**ABSTRACT**

Testing genetic purity of seeds is a mandate for testing authorities based on regulations of international seed testing associations. While delivery of pure seeds to growers should be of high confidence, certifying impure seeds would also be avoided with a minimum risk. This is achievable by lowering both producer and consumer error rates through designing an appropriate testing plan. Production of hybrid seeds is dependent on utilizing a pollination control system like Ogura which is a widely-used three-line system in rapeseed. Cytoplasmic male sterile (CMS) lines should be free from off-type fertile plants at a defined standard, as their adventitious presence (AP) above it result in losing genetic composition of consequent hybrid generation. We designed a multiplex PCR genetic assay for detection of fertile plants in sterile seed lots based on a deletion in promoter region of PPR-B gene of fertility restorer (*Rfo*) locus. Thus, presence of dominant allele (*Rf*-) in pools (taken from a composite sample) of CMS seeds (bearing recessive allele, *rfrf*) is a base for making a decision on accepting a pure lot or rejection an impure lot with low error rates. This genetic assay can be an alternative to control plots by reducing time and costs.

**Key words:** *Brassica napus* L., seed genetic purity, Ogura cytoplasmic male sterility.

## INTRODUCTION

Hybrid seed production in oilseed rape, rapeseed or canola (*Brassica napus* L.) relies on several pollination control systems (PCS), of which Ogura CMS is the most commercially used and extensively studied three-line system in plants (Chen et al. 2014). First studies of this system were carried out in Japanese radish by Ogura (Ogura 1968), hence the name, with the aim of producing hybrid seeds. Afterward, both CMS and nuclear restorer genes (*Rfo*) were introduced from radish (*Raphanus sativus* L.) to rapeseed that cause sterility and fertility phenotypes in CMS and

restorer inbred lines respectively. The responsible region in mitochondrial genome whose expression results in male sterility is a 417-bp open reading frame (ORF), known as *orf138* (Yamagishi et al. 2001). On the other hand, production of functional pollen is restored by nuclear *Rfo* locus that encodes three highly similar pentatricopeptide repeat (PPR) genes (PPR-A, -B, -C) (Desloire et al. 2003). Among the gene cluster, PPR-B plays the main role of restoring fertility in pollen plant and subsequent $F_1$hybrid generation by suppression of ORF138 translation in tapetum (innermost cell layer) of young anthers (Uyttewaal et al. 2008).

Commercial seed production of $F_1$ hybrid rapeseed in three-line PCS is by crossing A-line (CMS seed parent plant) with R-line (fertility restorer or pollen plant) in the field. A-line plant itself is propagated by crossing with its cognate isogenic maintainer line (B-line) which has a normal (fertile) cytoplasm. Cross-pollination is largely performed by insect activities (placing bee hives in seed production field), wind and artificially shaking male parent rows. Prior to production of $F_1$ hybrid seed, isolation of fields of A- and R-line is a key factor for conserving genetic purity of lines.

Checking varietal purity of seeds which is of great importance for both producers and consumers (growers) is the responsibility of seed testing authorities by inspecting inbred line production in control plots. As regards inspecting A-line (CMS) seed production field, pollen shedding plants are considered as off-type. They might be fertile R-line or B-line which has unintentionally spiked with A-line during any step of line production or an A-line whose sterility has broken down due to environmental conditions.

According to seed schemes standard of the Organization for Economic Co-operation and Development (OECD), only 0.5% off-type plants are allowed in certified seed of CMS line as regulated in "OECD schemes for the varietal certification or the control of seed moving in international trade" in 2017 (available at: https://www.oecd.org/tad/code/oecd-seed-schemes.pdf). Notwithstanding visual assessment in the control plots, genetic detection of fertile seeds (off-type plants) in CMS seed lot can considerably save in time and field inspection costs. This could be achieved by adapting genetic purity test introduced by Remund et al. (2001) of estimating AP of transgenic (or other undesirable) traits in conventional seed lots. In this semi-quantitative test, seed pools which are taken from the composite sample (a mixture of seed samples) are investigated for presence or absence of a trait rather than testing individual seeds of sample that is impractical due to being time-consuming and expensive. Accordingly, unintentional presence of off-type fertile seeds in sterile seed lots could be detected based on *Rf* gene locus. In rapeseed, allele-specific primers based on PPR-B gene of *Rfo* has been introduced for distinguishing restorer and non-restorer lines (Hu et al. 2008). In petunia, it has shown that the recessive allele (*rf*) in CMS line is due to a 530-nt deletion in promoter region of *Rf-PPR592* locus (Bentolila et al. 2002). We developed a method based on assuming a similar deletion in promoter region of PPR-B gene that may likely cause nonfunctioning *Rfo* in restorer line (*RfRf*), hence producing recessive allele (*rfrf*) in CMS female line. Here we report a genetic assay for detecting AP of at least one fertile seed (bearing dominant allele) in pools of 100 sterile seeds (bearing recessive allele) through multiplex polymerase chain reaction (PCR) of PPR-B gene of *Rfo* locus and *orf138* gene. This is the first report of diagnosing recessive allele of *Rfo* based on PPR-B promoter region which could be implemented in AP testing plan in seed pools with a consequence for varietal purity of CMS seeds.

## MATERIALS AND METHODS

Individual seeds (film-coated) of CMS line (H1938-1) and R-line (R0542) of $F_1$ hybrid (Neptune) were used for the genetic assay. Moreover, leaves of off-type plants in taken from control plots of CMC line were used for dissecting their genetic composition regarding CMS and fertility restorer genes. The plant materials were bred and provided by Euralis Semences, France.

### Visual assessment of off-type plants

Pollen shedding plants as off-type were counted in control plots of CMS line in three locations *i.e.* Karaj (N: 35° 48′ 23.8′′; E: 50° 58′ 21.2′′), Buin Zahra (N: 35° 55′ 29.4′′; E: 50° 05′ 48′′), and Jovein (N: 36° 21′ 16.7′′; E: 57° 01′ 33.4′′).Visual inspection and estimation of off-type percentage were carried out according to "guideline for control plot tests and field inspection of seed crops" developed by OECD in 2012 (available at: http://www.oecd.org/tad/code/ControlPlotEN092012.pdf) at the flowering time when flowers of CMS line were pollen receptive.

### DNA Extraction from rapeseed leaf

DNA of off-type leaves was extracted according to hexadecyltrimethylammonium bromide (CTAB) method (Saghai Maroof et al. 1984) with modifications of doubling the concentration of extraction buffer (EB) materials and substituting 2-mercaptoethanol (0.1%) with dithiothreitol (0.4 g) freshly added to 100 mL of EB.

### DNA Extraction from seed pools

DNA of samples (pools of 100 film-coated seeds) was extracted on small scale basis using sodium dodecyl sulfate (SDS) method. Firstly, seed pools were crushed in 2-mL safe lock tubes containing 2 stainless steel balls in Tissuelyzer II (QIAGEN, Germany) for 3 minutes with a frequency of 30

shaking per second. After effective disruption of seeds, 1000µL of EB (0.5M Tris pH 7.5, 0.35M NaCl, 0.5% SDS) was added to the tubes, vortex mixed and heated at 68°C for 45 minutes in water bath. Then, the tubes were centrifuged in 5430 R (Eppendorf, Germany) for 10 minutes at 13,000 rpm. After that, the supernatant was transferred to a fresh tube and two-third volume of chloroform:isoamyl alcohol (24:1) was added to it. This step was repeated after vortex mixing and placing the tube for 15 minutes on ice, prior to centrifuging for 10 minutes at 13,000 rpm. The resulting supernatant again was transferred to a fresh tube. Two-volume of pre-chilled ethanol and one-tenth volume of 3 M sodium acetate were added to the tube. After gently inverting for 10 times, the tube was kept in freezer (– 20°C) for at least one hour. The tube then was centrifuged for 7 minutes at 7,000 rpm. After discarding the supernatant the resulting pellet was washed with pre-chilled ethanol 70% and centrifuged for 3 minutes at 7,000 rpm. Then discarding the supernatant was repeated and the pellet was air dried. Finally, the pellet was dissolved in 100µL of TE buffer (1M Tris, 0.5M EDTA, pH 8.0). Quality and quantity of DNAs were checked by Nanodrop® ND-1000 spectrophotometer (Thermo Scientific, the USA).

**Design of primers**

A pair of forward (ORF138-F: AGCTGGTTTTCTAACAACCAACATT') and reverse (ORF138-R: GTAATTTTGACACCTAGCCACCC) primers were designed for amplifying a 104 bp fragment of *orf138* (GenBank accession AB055435; Yamagishi et al. 2003) mitochondrial gene responsible for CMS. Moreover, two primer pairs were designed from nuclear fertility restorer gene, *Rfo* (GenBank accession AJ550021; Desloire et al. 2003). One primer pair (PromB-F: CTTGCCTTGGCCAGAGTATC, and PromB-R: CTCGATTCCTTCTCATTGCAG) targeted nucleotides 90131-90860 to amplify a 750 bp fragment of PPR-B promoter region (+104to –646 ATG start codon) of *Rfo* gene. The second pair (InterAB3F: CCGAGCTTGACGATAACCAT, and InterAB3R: CGATGGGTAGCAAGAGCTTC) was used for amplification of an 830 bp (nucleotides 87694-88523) intergenic PPR-A/PPR-B region. Primers were synthesized by Macrogen (South Korea).

**PCR conditions**

Multiplex PCR assays were performed in a volume of 15 µL containing 50 ng template DNA, 1X PCR Master Mix (0.025 units *Taq* DNA polymerase, 2mM $MgCl_2$, 0.2mM dNTPs) (CinnaGen, Iran), and 0.15 pmol (PromB/InterAB3) and 0.05 pmol (ORF138) forward and reverse primers. The amplification consisted of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C (denaturation) for 45seconds, 57°C (annealing) for 45 seconds and 72°C (extension) for 45 seconds, and a final extension at 72°C for 7 min. An electrophoresis unit (SCIE-PLAS, the UK) was used for resolving PCR products on a 2% agarose gel containing GelRed™ (Biotium, the USA). Image of gels was taken by G:BOX (SYNGENE, the UK) gel documentation system.

**Testing plan design and genetic impurity estimation**

An operating characteristic (OC) curve was designed by "Qual Plan Design" sheet of SeedCalc8 spreadsheet application (https://www.seedtest.org/en/statistical-tools-for-seed-testing-_content---1--1143--279.html) for AP testing of dominant *Rf* allele in a randomly taken sample of 4800 CMS seeds (48 pools containing 100 seeds) bearing recessive *rf*/*rf* locus. Selection of 48 pools was according to 2×24 adapter set of Tissuelyzer II (QIAGEN, Germany) and fixed-angle rotor FA-45-24-11-HS (for 24 × 1.5/2.0 mL Tubes) of 5430 R centrifuge (Eppendorf, Germany). Maximum number (0.5%) of pollen shedding plants (off-type) allowed in certified seed of CMS line was considered as lower quality limit (LQL). Accordingly, acceptable quality level (AQL) was hypothetically considered as 0.2% to meet LQL threshold by seed producer through multiplication practices. In addition, false positive and negative rates of the genetic assay were inputted as 2 and 1% in testing plan respectively. Genetic impurity (*GI*) of the seed lot was estimated by $GI = \left(1 - \left(1 - \frac{d}{n}\right)^{\frac{1}{m}}\right) \times 100$ formula given in "% GM Estimate" sheet of SeedCalc8, wherein *d* is number of deviant (positive) pools, *n* is number of pools, and *m* is number of seeds per pool.

**RESULTS**

**Multiplex genetic assay**

While the amplification of a 104-bp fragment of *orf138* mitochondrial gene with ORF138 primer in A-line (CMS) was expected, an amplicon with similar size was also detected in R-line (fertility restorer) indicating that it bears a sterile cytoplasm. Accordingly, all plant materials we studied contained sterile (S) cytoplasm; however, the sterility is suppressed by nuclear *Rfo* genes that results in fertile plant in R-line and subsequent $F_1$ hybrid generation. Multiplex PCR of *orf138* and PPR-B promoter region (+104 to –646 ATG start codon) of *Rfo* gene could distinguish CMS line (H1938-1) and R-line (R0542) with respective ORF138 and PromB primers. This suggests that a deletion in promoter region of PPR-B gene results in absence of 750-bp amplicon and consequently bringing recessive allele (*rfrf*) in CMS lines (Fig.

1). Nevertheless, amplification of an 830-bp fragment of intergenic region PPR-A/PPR-B genes with InterAB3 primer demonstrated that the *Rfo* gene cluster is available in CMS line as well as restorer lines (data not shown). We tested presence and absence of Prom-B amplicon respectively in other CMS and restorer lines of Ogura-based hybrid seeds *i.e.* Natalie, Hydromel, Betty, Saphir (Euralis Semences, France) and Hyola 50 and Hyola 4815 (Pacific Seeds, Australia), though, an amplicon with a larger size was detected in some restorer materials than expected 750-bp fragment.

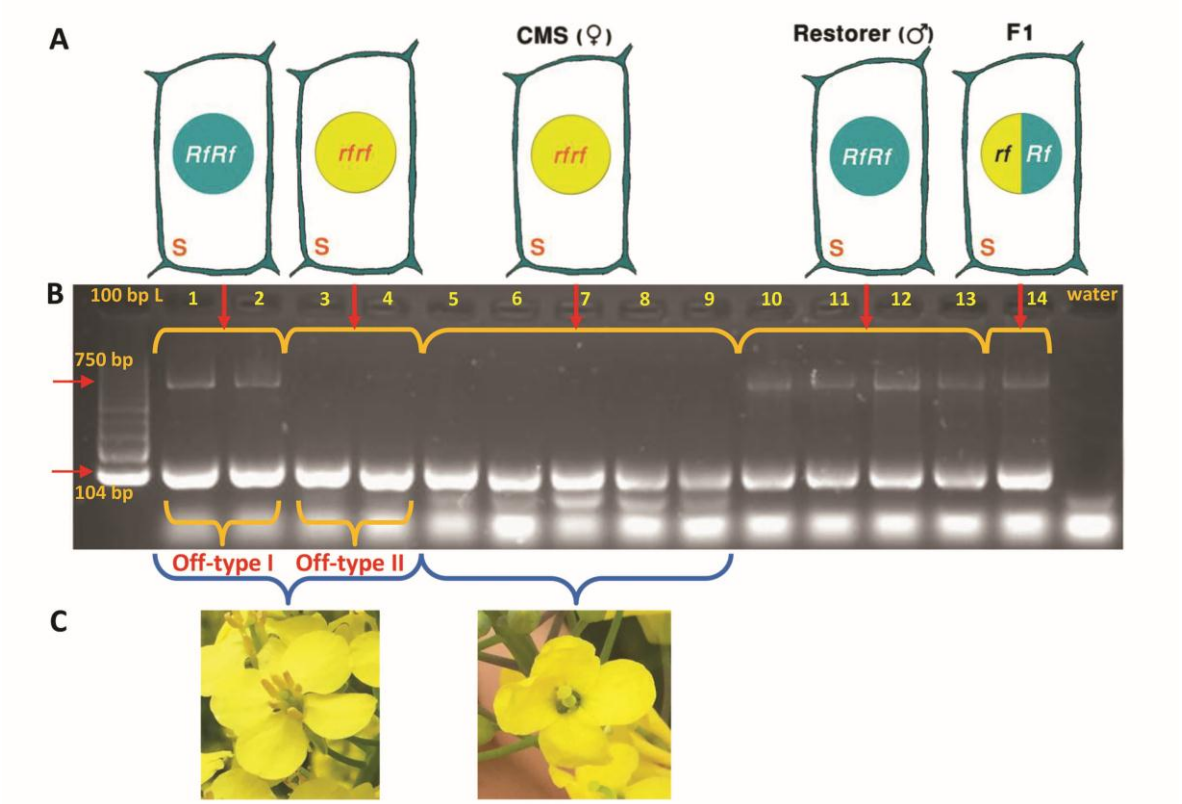


**Fig 1.** Agarose gel demonstrating results of the genetic assay for dissecting genetic composition of $F_1$ hybrid seed components. (A) Schematic figures of plant cells with similar sterile (S) cytoplasm but different alleles for fertility restorer (*Rfo*) gene. Cytoplasmic male sterile (CMS) female and fertility restorer lines of $F_1$ hybrid bear recessive (*rfrf*) and dominant (*RfRf*) alleles respectively. (B) Multiplex PCR of a l04-bp of mitochondrial *orf138* with a 750-bp of nuclear PPR-B gene at *Rfo* locus conferring CMS and restored fertility respectively. A deletion in promoter region of PPR-B results in recessive allele in five individuals of H1938-1CMS line (Lanes 5-9), while functional gene in four individuals of R0542 restorer line (Lanes 10-13) and $F_1$ generation (Lane 14) restores fertility. Amplication of these two loci in DNA (extracted from leaf) of pollen shedding plants differentiates two kind of off-types. One type (I) with functional PPR-B gene (Lanes 1-2) indicates their unintentional admixture in CMS seed lot. Other type (II) (Lanes 3-4) with recessive allele (*rfrf*) shows a CMS plant whose sterility has broken down due to environmental conditions in the field. (C) Phenotypes of restored fertility (left) and cytoplasmic male sterility (right) flowers of rapeseed.

**Genotyping pollen shedding plants**

Estimated number of off-type plants in control plots conducted in three locations exceeded the OECD seed purity standard (0.5%) for rapeseed hybrid varieties. Accordingly, percentage of pollen shedding plants (off-type) was 2% in Karaj, and 2.5% in Buin Zahra as well as Jovein. Genotyping off-type plants (taken from control plots of CMS line) showed that they differ in *Rfo* locus. One type (I) had a 750-bp amplicon of PPR-B promoter region indicating possibly cross contamination of dominant allele (*Rf*-) in the seed lot of CMS line due to not keeping field isolation standard. On the other hand, this amplicon was not present in PCR product of other off-type (type II), though amplification of 104-bp fragment of *orf138* indicated its S cytoplasm. This rejects the hypothesis of admixture of fertile B-line (with N cytoplasm) in A-line seed lot which may occur during multiplication process of A-line with maintainer B-line. Therefore, off-type (II) is an A-line seed parent plant whose sterility has broken down owing to effect of environmental conditions.

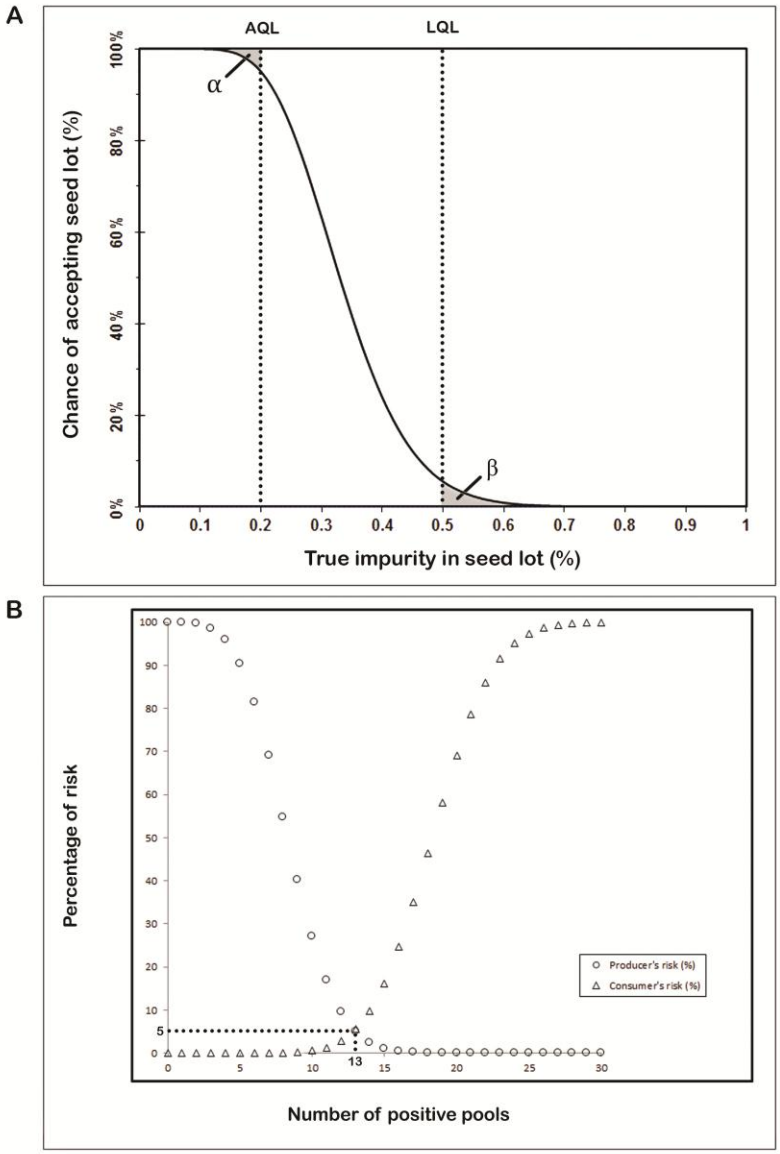


**Fig 2.** Operating characteristic (OC) curve for 48 seed pools tested and relationship between numbers of positive pools with percentage of risk. (A) OC curve based on designed testing plan for 4800 seeds in form of 48 pools of 100 sterile seeds. Lower quality limit (LQL) and acceptable quality level (AQL) were considered as 0.5 and 0.2% respectively with inputting 2% false positive and 1% negative rates. Based on this plan, producer (α) and consumer (β) risks were maintained at minimized levels of 5.05% and 5.5% respectively. (B) Relationship between number of positive (deviant) pools detected in genetic assay of PPR-B gene in sterile seed pools and percentage of producer's and consumer's risk. The corresponding α and β risks are maintained at 5% when the number of deviant pools are permitted to 13.

**Designing a good testing plan**

Based on designing an optimal OC curve for 48 pools of 100 seeds with considering 0.5% LQL (five off-type plants in 1000 plants) and 0.2% AQL (two off-type plants in 1000 plants) , producer (α) and consumer (β) error rates were maintained at minimized levels of 5.05% and 5.5% respectively (Fig. 2A). Consequently, a good AP testing plan was designed wherein the chance of rejecting a CMS seed lot that has an actual purity at the considered AQL and chance of accepting an impure CMS seed lot exceeding pre-determined impurity threshold (LQL) is of an acceptable risk. In order to assign the rejection criterion, we plotted number of positive (deviant) pools (contaminated with dominant *Rf*-allele) against the corresponding consumer's and producer's risk. Accordingly, we accept the CMS seed lot if the number of positive pools does not exceed 13 seed pools (cut-off point) with targeted 95% confidence level for both α and β risks (Fig. 2B).

**Testing for adventitious presence of off-type fertile seeds in CMS lots**

The SDS method yielded (on average) 500 ng/µl DNA from each pool of 100 seeds, while its quality

did not influence by materials (including colorant) used in film coating of seeds. Multiplex PCR of PromB and ORF138 in 48 pools of 100 CMS (A-line) seeds resulted in detection of deviant pools exceeding tolerable cut-off point (13 positive). On the other hand, based on detection of 36 positive pools out of 48, the genetic impurity of the seed lot was estimated as 1.38% using *GI* formula. This value was in congruent with impurity level (2.3% on average) of control plots across three locations. However, the discrepancy (0.92%) between values of the genetic assay and visual inspection is due to incapability of our method in identification of off-type II plants, those with *rfrf* allele. As these plants have the recessive allele (due to a deletion in promoter region of PPR-B gene), they are an A-line (CMS) plants with broken down sterility in the field. However, the genetic composition of pollen shedding plants as off-type plants (type I/II) in CMS line control plots is not a matter for field inspector, as they AP result in genetic impurity in the successive $F_1$ hybrid generation.

## DISCUSSION

Conduction of control plots is considered to be time-consuming and expensive due to inspection costs. Particularly for late summer sown trials, it takes several months to achieve inflorescence time when field inspection is carried out. Moreover, these trials are subject to environmental factors where sterility of seed parent (CMS line) could be broken down. Thus, a decision concerning accepting a genetically pure seed lot may be influenced by these factors, though inspector's estimation is irrelevant to genetic composition of off-type plants. However, once the number of off-type plants, including pollen shedding plants exceeds the purity standard (99.5%) a decision on rejecting the plot would be inevitable. The multiplex assay helped in distinguishing two kinds of off-type plants detected in control plots. Based on amplification of target loci in leaf-extracted DNA of off-type plants, we assigned off-type (I) a restorer line that was unintentionally spiked with CMS seed lot. On the other hand, the other type (II) indicated a CMS plant whose sterility has broken down due to environmental conditions in the field.

We implemented the introduced multiplex genetic assay in a testing plan for 48 seed pools comprised of 100 CMS seeds. The discrepancy between estimated values of off-type plants in the genetic assay (using *GI* formula) and visual inspection indicated that emergence of off-type (II) plants resulted in over-estimation of off-types in the field. Therefore, the estimated values of genetic assay are close to real values as it can only detect off-type (I) bearing functional fertility restorer gene. In order to achieve this aim, both producer's ($\alpha$) and consumer's ($\beta$) errors were maintained at 5% reasonable levels in semi-quantitative test of 4800 CMS seeds. Thus, a seed testing authority will reject a CMS seed lot with 0.5% impurity only 5% of the time.

This is the first report of implementing AP testing of undesirable *Rfo* trait in sterile seed lots, as introduced for transgenic traits in conventional seed lots (Remund et al. 2001). Moreover, this is the first report of diagnosing recessive allele of *Rfo* based on PPR-B promoter region, despite previously reported allele-specific primers for discriminating restorer and non-restorer lines (Hu et al. 2008). Utilization of introduced multiplex PCR genetic assay in seed pools can considerably reduce in inspection costs, while be an alternative method for estimation of off-type plants in control plots in much lower time. This assay could also be deployed by seed testing authorities for control of seed moving in international trade as regulated by international seed associations.

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