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Research Paper

RAPID DETECTION OF GENETICALLY MODIFIED ORGANISMS IN COTTON SEEDS BY REAL TIME PCR

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The ability to detect presence or absence of foreign gene(s) relies on high yields of pure DNA samples. Cotton seeds contain polyphenols, polysaccharides and other secondary metabolites which often interfere during DNA isolation. Here we present optimization of DNA isolation and Real Time PCR conditions for the detection of GMO in cotton seeds (*Gossypium* spp.). The modified CTAB protocol includes addition of sodium bisulfite in combination with higher CTAB concentration and precipitation of DNA with 5 M NaCl in combination with chilled ethanol increased the solubility of polysaccharides and polyphenols. This protocol is devoid of use of expensive chemicals such as Proteinase K and RNase etc. We also optimized Real Time PCR conditions such as annealing temperature and concentration of DNA for the detection of CaMV 35S target gene. Four samples out of five were found to be GM positive with reference to target gene.

Keywords: Cotton seeds, DNA isolation, GMO analysis, CaMV35S promoter, Real Time PCR

INTRODUCTION

Genetically modified organisms (GMO's) contain specific traits/ genes which are inserted in to organisms to improve their property which does not generally occur by natural mating (Anklam *et al*., 2002). Such genetically modified crops have made a vital contribution to food, feed and fibre security in today's world. By the end of year 2010, the global area of GM plants was 148 m ha which was just 1.7 m ha in 1996. Now a day's around forty five GM crops are available of which soya, cotton, maize and potato are major GM crops. As far as India is concerned, GM cotton covers around 10.0 m ha of land and is the only dominant crop produced on commercial basis. BT cotton was the first introduced by a USA based company Monsanto in collaboration with Maharashtra Hybrid Seed Company (MHYCO) and was commercialized in 1990's at the global level and officially approved for sale in India in 2002. Cotton is an important crop for textile industry and is the food and feed source of many areas of the world. It was developed to reduce heavy resilience on pesticide; *Bacillus thurengenesis* naturally produces a chemical harmful to a small fraction to insects, larve of moths, butterflies, flies and

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harmful to other forms of life. The gene coding for BT toxin has been inserted in to cotton, causing cotton to produce a natural insecticide in its tissues. Cotton contains gossypol, a toxin that makes it inedible. However scientists have silenced the gene that produces a toxin thereby making it a potential food crop.

In genetically modified crops most common recombinant elements are Cauliflower Mosaic Virus (CaMV 35S promoter) and NOS terminator sequences (Lipp *et al*., 1999) both of which are transcription regulating sequences (Odell *et al*., 1985). As the demand to detect transgene is vastly increasing due to introduction of new GM crops, the development of rapid detection protocols have also become much more challenging and mandatory.

A series of analytical tools based on detecting DNA, RNA and Protein are available to differentiate between GM and non GM crops. Among DNA based methods, Polymerase Chain Reaction (PCR) is the most widely used method in research as well as in commercial laboratories because of its high sensitivity and specificity (Anklam *et al.,* 2002 and Ahmed, 2002). Real Time PCR is preferred over conventional PCR as it allows monitoring of reaction in real time through fluorescence (Heid *et al*., 1996) plus no post agarose gel detection is required in Real Time PCR which used SYBR green dye I chemistry. This has a high affinity towards all double stranded DNA (Morrison *et al*., 1998) and a melting curve is generated at the end of reaction allowing post PCR identification in expected target as well as in closely related targets. Another advantage is that it is cost effective as no dye labeled oligonucleotide probes is required (Terry and Harris, 2001).

Cotton (*Gossypium* spp.) being a tree is a very difficult plant for DNA isolation from leaves or from seeds due to presence of phenolic compounds that generally interfere with quality and quantity of DNA by making it unstable for downstream processes such as RFLP, RAPD and PCR etc. There are many protocols available for isolation of genomic DNA from cotton seeds, but all these are laborious, time consuming and costly. For commercial basis a rapid and pure isolation of genomic DNA is a prerequisite. Taking this into account, it was decided to standardize a rapid and cost effective method for isolation of genomic DNA from cotton seeds and optimizing Real Time PCR conditions for qualitative detection of target gene (CaMV 35S promoter) in cotton seeds.

MATERIALS AND METHODS

Sample Collection

Five cotton seed samples were collected from different regions of Maharashtra.

Chemicals and Other Reagents

Extraction buffer containing 2.5% CTAB, 1.8M NaCl, 0.1M Tris-HCl (pH-8.0), 0.02M EDTA.$2H_20$ (pH-8.2), 0.35 M Sodium bisulfite and 0.1% β-mercaptoethanol (added just before the use)

Phenol: Chloroform: isoamyl alcohol (25:24:1).

5M NaCl

Chilled distilled ethanol

0.1X TE buffer containing 10mM Tris (pH-8) and 1 mM EDTA. $2H_20$ (pH-8)

Primer Information

Primers were synthesized from Sigma Aldrich, USA. All primers were diluted to the working concentrations 10pmol/l with sterile deionized water. The sequences of the primers are given in Table 1.

Table 1: Primers used for Real Time PCR study

Oligo Name	Sequence	GC%
CaMV forward	GCTCCTACAAATGCCATCA	47%
CaMV reverse	GATAGTGGGATTGTGCGTCA	50%
Sah 7 forward	AGTTTGTAGTTTTGATGTTACATTGAG	32%
Sah 7 reverse	GCATCTTTGAACCGCCTACTG	52%

DNA Isolation

GMO CRL Protocol

1. Take 6 gms of cotton seed powder and add 25 ml of CTAB extraction buffer containing 0.5ml of β-mercaptoethanol and 0.25ml of Proteinase K. Mix the content by inversion. 2. Incubate at 55^{0}C for 60 mins with intermittent shaking. Cool the tube after incubation. 3. Add 20 ml of Phenol: Chloroform: Isoamylalchohol (25:24:1) and mix gently by inversion. 4. Centrifuge at 13,000 g for 10 mins at room temperature. 5. Collect the aqueous layer and repeat step 3 and 4 twice. 6. To the collected aqueous layer add 2 volumes of chilled isopropanol and gently mix by inversion. 7. Allow the DNA threads to appear. 8 Spin the DNA at 5000 rpm for 10 mins at 4^{0}C. 9. Remove the supernatant and allow the pellet to dry overnight 10. Next day dissolve the pellet in 2ml of TE buffer and store at -20^{0}C. 11. Add 50µl of RNase and incubate at 37^{0}C for 30 min. 12. To extract DNA, add 4 ml of Chloroform: Isoamylalchohol (24:1) and mix the content vigorously. 13. Centrifuge at 10000 rpm for 10 min at room temperature. 14. To the upper layer, add half volumes of 7.5M ammonium acetate and add 2 volumes of 100% ethanol. 15. Allow the DNA to appear again and spin the content at 4^{0}C for 10 min at 5000 rpm. 16. Throw the supernatant and allow the pellet to dry and next day add TE buffer.

Standardized Protocol

1. Weigh 30 mg of finely grinded cotton seed powder in a 2 ml microcentrifuge tube. 2. Add 250 µl of extraction buffer and mix well with gentle inversion. 3. Incubate the content at 60^{0}C for one hour with intermittent shaking after 10 min. 4. Centrifuge at 12,000 rpm for 20 min at 25^{0}C. 5. Collect the aqueous layer and add equal volume of phenol: chloroform: isoamylalchol (25:24:1) and mix well by gentle inversion. 6. Centrifuge at 12,000 rpm for 20 mins at 25^{0}C. 7. Repeat steps 5 and 6 twice. 8. To the aqueous layer, 0.5 volumes of 5 M NaCl was added followed by 2 volumes chilled distilled ethanol and mix by gentle inversion. 9. Allow the DNA threads to appear. 10. Spin the content at 4^{0}C for 10 min at 5000 rpm. 11. Throw the supernatant and allow the pellet to dry overnight. 12. To the pellet, add 150 µl of 0.1X TE buffer and store at –20^{0}C till use.

Agarose Gel Electrophoresis and DNA Purity

The genomic DNA was resolved on 0.8% agarose gel in 1X TAE buffer with internal ethidium bromide staining and electrophoresis was carried out at 65V for 2 hrs and visualized under UV transilluminator. DNA purity was checked by measuring the absorbance at 260 nm and 280 nm.

Real Time PCR

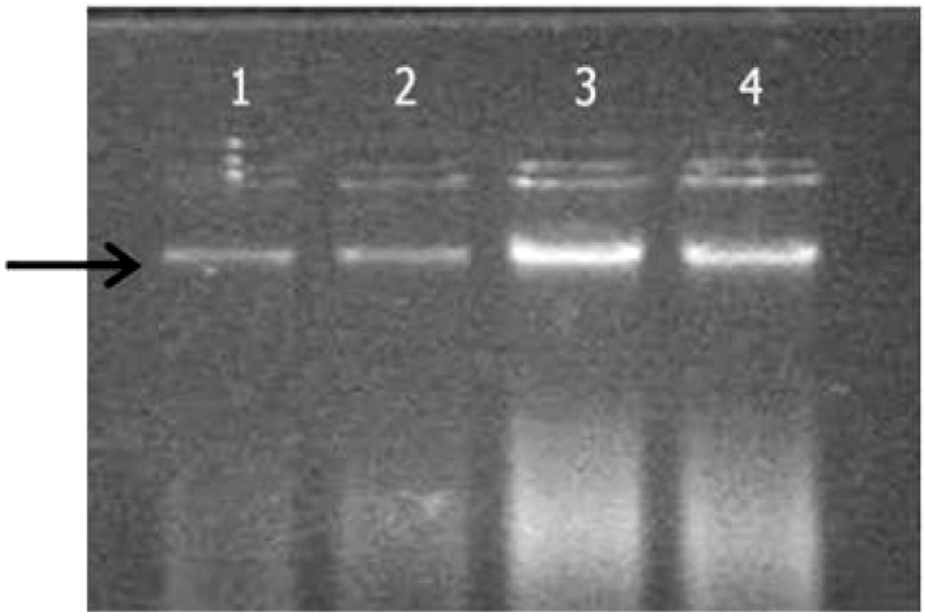
In order to check whether the extracted was

suitable for GMO detection, Real Time PCR was performed using SYBR Green Master Mix**.** Real Time PCR analysis was carried out in 7300 Real Time PCR System (Applied Biosystems, USA). All the amplification reactions were performed in 96 well plates. Individual reactions contains 12.5 µl of 2.5X SYBR Green Master mix, 5 µl of template DNA, 1 µl of each forward and reverse primer and finally volumised to 25 µl using sterile Mili-Q water. Negative template control was also run along with DNA samples to avoid the detection of false positive and false negative. For positive control we used standard cotton seed powder DNA (0.1%) (ERM-BF413b) purchased from European Union (EU) Joint Research Center, Fluka. PCR conditions involved an initial denaturation for 5 min at 95^0C, followed by 40 cycles of 95^0C for 15 sec, 60^0C for 1 min and 72^0C for 2 min. Finally reactions were held at 72^0C for 5 min. The amplification products were electrophoresed on 2% agarose gel for 1 hr at 100 volts with internal ethidium bromide staining to confirm the desired product.

RESULTS AND DISCUSSION

Isolation of good quality of genomic DNA from cotton seeds was very difficult due to presence of phenolic compounds. During tissue homogenization, these compounds oxidize and thus irreversibly bind with proteins and other nucleic acids. This irreversible binding produces a gelatinous pellet with brownish color which is hard to separate from organelles and thereby making DNA unstable for downstream processes. Most procedures for DNA isolation from plant species containing high levels of phenolic compounds are modified versions of standard protocols. In our study, GMO-CRL method reported earlier for isolation of genomic DNA from modified cotton seeds did not work well. Though we could able to isolate DNA but the pellet was brownish having gel like appearance made it difficult to dissolve in 0.1X TE buffer. Electrophoresis pattern showed presence of RNA along with proteins and other impurities (Figure 1, lane 3 and 4). We also noticed that yield was very less though starting material was around 3 gms. $A_{260/280}$ ratio was 2.4 with GMO-CRL protocol indicating interference of RNA. Hence we have devised a simpler procedure that used sodium bisulfite as a reducing agent and extraction buffer based on the CTAB method. We observed that addition of 0.35 M sodium bisulfite to extraction buffer drastically improved the DNA quantity and quality, avoiding contamination and browning of the pellet by phenolic compounds. The purity decreased when we used lower concentration (0.15 M) of sodium bisulfite and with higher concentration (1M) the yield was less probably because 1M sodium bisulfite fulfills a role of osmoprotectant. In our case we just used 50 mg of cotton seed powder as the starting material which was very less as compared to GMO-CRL method. During precipitation of DNA, we used chilled ethanol which improved the yield. It has been reported that higher concentrations of NaCl along with ethanol increases the solubility of polysaccharides and phenolic compounds (Fang *et al*., 1992; Sahasrabudhe and Deodhar, 2010). Thus we could able to isolate DNA free of RNA and protein contamination (Figure 1, lane 2) **(**depicted with black arrow). The freer the DNA is from contaminants easier it is to resuspend the pellet. Disadvantage in GMO-CRL protocol was we need to re precipitate DNA again after RNase treatment which makes it time consuming plus there are chances of shearing of DNA on the other hand, our standardized method is fast, reliable and cost effective as no expensive chemicals such as RNase and Proteinase K are required.

Figure 1: Genomic DNA of Cotton Seeds Resolved on 0.8% Agarose Gel



Another advantage is that very less starting material is required for isolation of DNA (DNA yield was 1.015 µg with 30 mg of cotton seed powder.

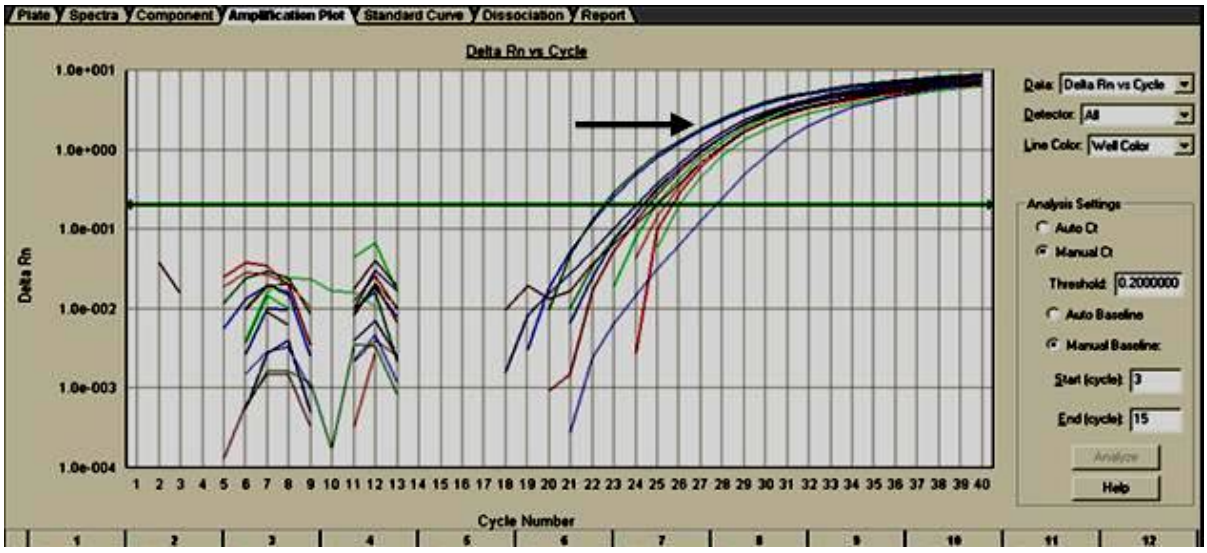
Real Time PCR Analysis

Initially we tried amplification pattern of DNA samples isolated from GMO-CRL protocol using Real Time PCR. We observed quenching in the amplification curve (Figure 2). The reason could be because of inhibition of *Taq DNA polymerase* due to the inhibitory components in the reaction mixture, proteins, RNA or polyphenolics contaminants present in the DNA sample. Also a small amplification curve was seen in the target gene of NTC. This might be because of the cross contamination in the adjacent wells or may be due to primer-dimmer formation. To overcome these difficulties, we then standardized our DNA extraction method to get pure and sufficient amount of genomic DNA. Our standardized method produced proper amplification curve (Figure 3). After standardizing DNA isolation protocol we then optimized various PCR cycle parameters such as annealing temperature and amount of DNA. We tried three annealing temperatures such as 55, 60 and 65^{0}C. At 55^{0}C there was a quenching seen in the amplification curve while at 65^{0}C there was no amplification but at 60^{0}C a proper curve was observed plus there was no amplification in NTC (Non template control) (Table 2, depicted with red arrow). For Real Time PCR analysis, we used 20, 40 and 80 ng of DNA samples. We found that at 20 ng there was no amplification curve but at 40 and 80 ng a proper amplification curve seen. Hence for further experiments we used 40 ng of DNA.

As mentioned earlier, we have tested five different cotton seed samples collected from five regions of Maharashtra. Interestingly among five samples, four samples were found to be GM positive since the amplification curve was observed in target as well as in endogenous gene (Figure 3). As we can see from the Table 2, Ct

Figure 2: Quenched Amplification Curve

Figure 3: Proper Amplification Curve



values of four samples were earlier than that of the standard GM (0.1%) target gene confirming that there was early amplification of target gene as compared to that of standard CaMV gene. Only sample one showed undetermined Ct value for target gene indicating complete absence of CaMV promoter gene but a proper amplification was observed in endogenous gene of the same sample confirming it as GM negative (depicted with black arrow). As the SYBR green dye I binds non specifically to any double stranded DNA, the measured florescence may have been contributed by non specific PCR products or by primer dimmers. In order to differentiate such artifacts from specific PCR products, a melt curve cycle was incorporated in to Real Time

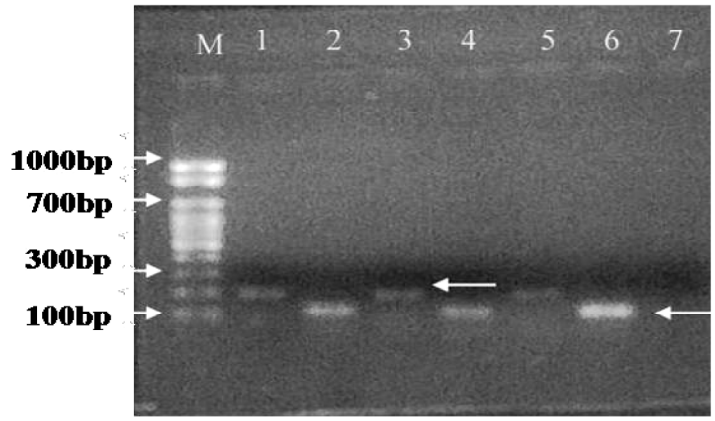
Table 2: Table Showing Ct Values and Tm for Each Test Sample

Plate | Spectra | Component | Amplification Plot | Standard Curve | Dissociation | Report

Well	Sample Name	Detector	Task	Ct	StdDev Ct	Qty	Mean Qty	StdDev Qty	Filtered	Tm
C3	cotton1	CAMV sybr	Unknown	Undet.						72.2 °C
C4	cotton1	CAMV sybr	Unknown	Undet.						69.5 °C
C5	cotton1	Seh 7	Unknown	23.49	0.266					75.1 °C
C6	cotton1	Seh 7	Unknown	23.09	0.266					75.1 °C
C7	cotton2	CAMV sybr	Unknown	24.97	0.046					80.6 °C
C8	cotton2	CAMV sybr	Unknown	25.04	0.046					80.6 °C
C9	cotton2	Seh 7	Unknown	23.40	1.222					74.4 °C
C10	cotton2	Seh 7	Unknown	25.13	1.222					74.1 °C
C11	cotton3	CAMV sybr	Unknown	22.81	0.061					80.3 °C
C12	cotton3	CAMV sybr	Unknown	22.90	0.061					80.3 °C
D1	cotton3	Seh 7	Unknown	23.01	0.480					74.8 °C
D2	cotton3	Seh 7	Unknown	22.33	0.480					75.1 °C
D3	cotton4	CAMV sybr	Unknown	25.43	0.641					80.9 °C
D4	cotton4	CAMV sybr	Unknown	24.53	0.641					80.9 °C
D5	cotton4	Seh 7	Unknown	24.08	0.171					75.1 °C
D6	cotton4	Seh 7	Unknown	24.32	0.171					75.1 °C
D7	cotton5	CAMV sybr	Unknown	26.24	2.142					79.9 °C
D8	cotton5	CAMV sybr	Unknown	23.21	2.142					80.9 °C
D9	cotton5	Seh 7	Unknown	25.72	0.856					74.4 °C
D10	cotton5	Seh 7	Unknown	24.51	0.856					74.4 °C
E1	STD	CAMV sybr	Standard	25.89	1.148					80.6 °C
E2	STD	CAMV sybr	Standard	25.33	1.148					80.9 °C
E3	STD	Seh 7	Standard	27.23	0.275					74.4 °C
E4	STD	Seh 7	Standard	26.84	0.275					74.8 °C
E5	NTC	CAMV sybr	NTC	Undet.						86.4 °C
E6	NTC	Seh 7	NTC	Undet.						88.2 °C

	1	2	3	4	5	6	7	8	9	10	11	12
A	U	U	U	U	U	U	U	U	U	U	U	U
B	U	U	U	U	U	U	U	U	S	S	S	S
C	N	N	U	U	U	U	U	U	U	U	U	U
D	U	U	U	U	U	U	U	U	U	U		
E	S	S	S	S	N	N	U	U	U	U	U	S

Figure 4: PCR Product Resolved on 2% Agarose Gel Lane 1, 3 and 5: Endogenous Gene Product, 2, 4 & 6: Target Gene Product and M: Molecular Marker



PCR. The melting temperature (T_m) is usually determined by products length, GC content, concentration of dye and specific template in the reaction tubes. Due to this non specific products normally melt at much lower temperature than that of specific products which are larger in size (Taverniers *et al*., 2005). In our case, melting temperature (T_m) of cotton one (Sample one) where Ct value was undetermined, was 72 and 69^0C which was less than that of rest of the other positive samples (Table 2).

After Real Time PCR cycle, agarose gel electrophoresis was carried out for the confirmation of deseired amplicons (Figure 4). It was observed that in case of target gene, an amplicon of 107 bp was prominent and that of endogeneous gene product size was ~190 bp in all tested samples confirming the proper Real Time PCR run.

CONCLUSION

PCR based methods for GMO detection appears to be the method of choice because of its high sensitivity and specificity. Genetic analysis of plants relies on high yield of pure DNA sample which is an essential prerequisite for the detection of GMO's in various crops. For this purpose we initially standardized rapid DNA isolation protocol devoid of expensive chemicals like RNase or Proteinase K.

To conclude we can say that sodium bisulphite CTAB based DNA isolation method and qualitative Real Time PCR analysis is suitable for rapid detection of GMO in cotton seeds on commercial scale. It is possible to detect the presence of transgene to an extent of 0.1%. As India will be implementing mandatory labeling on GM crops very soon, this qualitative approach will facilitate the labeling legislations.

REFERENCES

1. Ahmed F (2002), "Detection of Genetically Modified Organisms in Foods", *Trends Bioechnol.*, Vol. 20, No. 5, pp. 215-223.
2. Anklam E, Gadani F, Heinze P and Van den Eede (2002). "Analytical Methods for Detection and Determination of Genetically Modified Organisms in Agricultural Crops Plant Derived Food Products", *Eur. Food Res. Technol.*, Vol. 214, pp. 3-26.
3. Fang G, Hammar S and Rabecca R (1992), "A Quick and Inexpensive Method for Removing Polysaccharides from Plant Genomic DNA", *BioTechniques*, Vol. 13, pp. 52-56.
4. GMO CRL Protocol (2008), "Report on Validation of a DNA Extraction Method for Cotton Seeds and Grains, Method Testing and Single Laboratory Validation", *JRC European Commission*, Monsanto Company, pp. 1-14.
5. Heid C, Stevens J, Livak K and Williams P M (1996), "Real Time Quantitative PCR" *Genome Res.,* Vol. 6, No. 10, pp. 986-994.
6. Lipp M, Brodmann P, Pietsch K, Pauwels J and Anklam E (1999), "IUPAC Collaboration Trial Study of a Method to Detect Genetically Modified Soybeans and Maize in Dried Powder", *J. AOAC Int*, Vol. 82, pp. 923-928.
7. Morrison T, Wies J J and Wittwer C T (1998), "Quantification of Low Copy Transcripts by Continuous SYBR Green I Monitoring During Amplification", *Biotechniques*, Vol. 24, No. 6, pp. 954-962.
8. Odell J, Nagy F and Chua N H (1985), "Identification of DNA Sequences Required for the Activity of the Cauliflower Mosaic Virus 35S Promoter", *Nature*, Vol. 313, pp. 810-812.
9. Sahasrabudhe A and Deodhar M (2010), "Standardization of DNA Extraction and Optimization of RAPD-PCR Conditions in *Garcinia indica"*, *Int. J. Bot.*, Vol. 6, No. 3, pp. 293-298.
10. Tavernier I, Van Bockstaele E, Vaitilingom M, Milcamps A, Van den Eede G and De Loose M (2005), "Event Specific Plasmid Standards and Real Time PCR Methods for Transgenic Bt11, Bt176 and GA21 Maize and Transgenic GT73 Canola", *J. Agric. Food. Chem.,* Vol. 53, pp. 3041-3052.
11. Terry C and Harris N (2001), "Event specific detection of Roundup Ready Soya Using Two Different Real Time PCR Detection Chemistries", *Eur. Food Res. Technol.*, Vol. 213, pp. 425-431.