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Optimization of different DNA extraction methods for the molecular detection of resistance genes

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ABSTRACT

Tomato spotted wilt virus (TSWV) and Pepper mild mottle virus (PMMoV) not only cause economic yield losses but also limit production for pepper plants. Resistant genes are the only reliable management strategies to control such viral diseases, but these viruses are able to overcome such resistance mechanisms. Therefore, novel resistance genes should be used to control the TSWV and PMMoV diseases. For molecular detection of resistance genes, DNA quality and purity are extremely vital to obtain proper results. This study aims to identify the best extraction method using resistance gene markers from different leaves of pepper plants. PCR analyzes revealed that fresh first real leaf gave reliable DNA quality with the CTAB DNA extraction method and then the Dellaporta extraction method which yielded lower concentrations. Although the commercial DNA extraction method offered convenient results within PCR analyzes, it is cost effective for molecular breeding such as marker assisted selection programs in developing countries. The study has clearly addressed optimized DNA concentration, using different extraction methods, leaf samples from pepper plants and their storage conditions for ultimate results in breeding programs.

1. Introduction

Pepper (*Capsicum annuum* L.) holds significant economic importance in both Türkiyeand worldwide in terms of production and consumption. According to production volume, China, Mexico, and Türkiye are the top three producers worldwide (FAO 2018). National statistics from TUIK indicate that Türkiye produced 2636905 tons of pepper in 2020 (TUIK 2021). The pepper is consumed fresh and is also processed in the food industry as dried, pickled, paste, and spice products. The interest in identifying capsaicinoids found in hot peppers has recently increased due to their diverse applications, such as in topical creams, defensive sprays, and alternative medical treatments (Christo and Cauley 2009).

Pepper is an annual plant cultivated in temperate, warm climates, and various cultivars are grown in open fields and greenhouses depending on the climatic conditions. There are many cultivated types in Türkiye including Capia, sivri, charleston, dolma, California wonder, Maraş pepper, chili peppers, ornamental peppers, pickling peppers, jalapeño, and cherry types. However, viral diseases are among the major factors that significantly impact both yield and quality in pepper cultivation areas (Anandakumar et al. 2008). There are no effective chemical control methods against viral diseases; therefore, developing resistant varieties and implementing vector control measures are essential. Tomato spotted wilt virus (TSWV) is transmitted by thrips species such as *Frankliniella occidentalis* and *Thrips tabaci*, it is a tospovirus that rapidly spreads in pepper production areas. On the other hand, Pepper mild mottle virus (PMMoV) is a *tobamovirus*, and it is seedborne and transmitted through mechanical manners.

Currently, advance biological and biotechnological methods are widely applied in agricultural production. Through molecular genetic analysis of plant DNA, it is possible to identify plant genotypes and construct genetic maps similar to human genetic studies. Marker-assisted selection (MAS) is particularly important in plant breeding to detect the presence of disease resistance genes. One of the key technologies enabling this is the polymerase chain reaction (PCR), discovered by Mullis (1986). The PCR allows for the amplification of specific regions of DNA from any organism with known genomic sequences (Mullis et al. 1986).

Molecular markers linked with resistance alleles such as *L3*, *L4*, and *Tsw* genes are commonly used in resistance breeding against PMMoV and TSWV diseases. The SCAC568 CAPS marker has been associated with resistance to TSWV, it is used in breeding programs to distinguish between resistant and susceptible genotypes at the co-dominant level (İkten 2019). Resistance to *tobamoviruses* is effectively identified by markers linked to *L3* and *L4* genes. Among various studies, the most reliable primers for identifying *L4* genes associated with PMMoV resistance are AP-7/AP-8 primers developed (Matsunaga et al. 2003; Fidan and Barut 2019).

Traditional breeding methods consider quality and quantity of DNA extracted from different pepper plant populations can often limit PCR efficiency. The initial step in a successful PCR

reaction is the isolation of total nucleic acids. Commercial DNA extraction kit has been used in 90% of resistance screening studies against TSWV in Turkish pepper varieties. While these commercial kits provide fast and clean DNA extraction, they are costly for large-scale sample processing. Among alternative methods, the CTAB method (Doyle and Doyle 1987) is widely used; however, the use of chloroform in this method poses serious health risks such as dizziness, fatigue, respiratory irritation, and long-term damage to liver and kidneys. Moreover, the phenological stage of the sampled plant, storage conditions, and sample quantity also influence DNA extraction qualities. Therefore, it is essential to employ low-cost, less hazardous chemicals during DNA isolation. This necessity has prompted us to search for reliable extraction protocols (Aka-Kaçar 2003; Aleksić et al. 2012).

The aim of this study was to optimize CTAB, Dellaporta, and commercial kit-based DNA extraction methods for the isolation of total nucleic acids intended for use for PCR applications, and to evaluate the impact of DNA quality on PCR efficiency using molecular marker analysis across different pepper varieties.

2. Materials and Methods

In this study, two different internationally developed protocols, modified CTAB and Dellaporta, and commercial kit, GeneMark Plant DNA Purification Kit (APS Lifetech, Netherland), were used to extract total DNA. In PCR gene screening, SCAC568 was used in the resistance analysis against TSWV, and AP-7/AP-8 markers were used in the resistance analysis against PMMoV respectively. For each gene analysis, 3 different commercial pepper varieties from 7 different segments were studied where these varieties were *L4* resistant commercial variety (A), *Tsw* gene containing variety (B) and susceptible pepper variety (C) to both TSWV and PMMoV.

For each commercial variety, 3 individual pepper seeds were sown in vials containing 1 volume (v) peat: 1 v perlite: 1 v vermiculite mixture. The vials were placed in acclimatization chambers in total darkness at 26ºC for 48 hours. After this period, the acclimatization chamber was adjusted to 16 hours of light and 8 hours of night period at 26ºC. For analyzes, 39 plants, with cotyledons and first 5 true leaves emerging after the cotyledons were collected and stored at -20ºC. The leaf samples were either used or stored over 90 days at -80ºC for DNA extractions. Detailed DNA isolation protocols and preparations of extraction solutions are provided below, and the obtained DNA samples were statistically analyzed by T test (Santos et al. 2010).

2.1. The CTAB extraction protocol

In the CTAB method, 5 ml CTAB + 5 µl β-Mercaptoethanol was mixed and used in the crushing stage of the samples. A 500 mg leaf sample was first crushed with 15 µl of CTAB solution. The amount was completed to 500 µl and transferred to microtubes. The tubes were incubated at 65 ºC for 2 hours at 350 rpm. Eppendorf tubes were cooled for 5 minutes, 500 µl of 24 chloroform: 1 isoamyl alcohol solution was added to them, they were turned upside down and centrifuged at 13000 rpm for 15 minutes, and the supernatant liquid was transferred to new microtubes. A 500 µl of 24 chloroform: 1 isoamyl alcohol was added to the microtubes again and the centrifugation process was repeated. The samples were transferred back to new tubes then supernatant liquid was drawn as cleanly and carefully as possible and transferred to new tubes. Isopropanol alcohol was added as much as the drawn volume and turned upside down. The microtubes containing the samples were kept at -20ºC overnight. The next day, the samples were centrifuged at 13000 rpm for 20 minutes. The supernatant was carefully poured off and cold 70% alcohol was added to the samples and centrifuged at 13000 rpm for 5 minutes and the supernatant liquid was poured off. 500 µl of ethanol was added to the samples again and the process was repeated. After the last liquid part was poured off, the samples were left to dry. After the samples dried, they were diluted with 100 µl of ddH_2O and kept at +4ºC for one day (Doyle and Doyle 1987).

2.2. The Dellaporta extraction protocol

In this method (Dellaporta et al. 1983), a 500 mg sample was ground in 1.2 ml of extraction buffer including 100 mM Tris, 50 mM EDTA, 500 mM NaCl, 10 mM β-Mercaptoethanol. A volume of 600 µl from the homogenized sample was transferred into 1.5 ml microtubes. Subsequently, 70 µl of 10% SDS was added, and the tubes were incubated at 65°C for 10 minutes. The tubes were inverted twice during incubation then samples were allowed to cool for 5 minutes. 200 µl of 5 M potassium acetate was added to each tube and the tubes were placed on ice for 10-30 minutes. During this period, the tubes were inverted twice then the samples were centrifuged at 10000 rpm for 10 minutes at 10 °C. From the supernatant of each centrifuged tube, 600 µl was carefully transferred into new tubes. An equal volume (600 µl) of ice-cold 96% ethanol was added, and the tubes were inverted a few times. The samples were placed overnight in a−20°C deep freezer. The next day, the samples were centrifuged at 10000 rpm for 10 minutes to precipitate the nucleic acids. The supernatant was carefully removed without disturbing the pellet. The remaining pellets in the tubes were left to air dry for 10-15 minutes. Finally, 200 µl of sterile distilled water was added onto dried pellets, and the tubes were incubated at 37°C for 15 minutes to completely dissolve the nucleic acids.

2.3. Commercial kit DNA extraction protocol

DNA isolations were performed from collected plant samples according to the protocol recommended by the manufacturer of the Thermo Scientific GeneJET Plant Genomic DNA Purification Mini Kit (Lot No: 2921857). After completion of the isolation procedures, the quality of the DNA was assessed by staining the samples with loading dye and preparing them in a 1.5% agarose gel made with TAE buffer solution. A volume of 6 µl from each sample was loaded into individual wells of the gel, and electrophoresis was carried out at 100 V for 45 minutes. The gel was stained with ethidium bromide and visualized under a UV transilluminator. To determine the purity and concentration of the nucleic acids following the isolations, spectrophotometric measurements were conducted for each sample, then DNA concentrations were adjusted to 50 ng μl^{-1}.

In the PCR reactions, a total volume of 50 µl reaction mixture was used, containing genomic DNA at a concentration of 50 ng μl^{-1}, 12 µl of ddH_2O, 24 µl of Ecotaq, and 2 µl each of forward and reverse primers (Table 1). The thermal cycling program in the PCR machine (Thermo Scientific, Germany) as follows: an initial denaturation at 95°C for 3 minutes then 30 cycles of denaturation at 95°C for 50 seconds (s.), annealing at 57/58°C for 45 s and extension at 72°C for 50 s with a final extension step at 72°C for 10 minutes.

For each plant sample, three different isolation methods and three different sampling conditions were applied, resulting in 12 distinct outcomes per sample. A total of 156 data were obtained

Table 1. The markers used to amplify TSWV and PMMoV resistance genes from isolated DNA samples in PCR analyses

Marker Name		Primer Sequence	Amplicon Size (Bp)	Annealing Temperature
SCAC568	Forward	5'GTGCCAGAGGAGGATTTAT3'	568	57°C
	Reverse	5'GCGAGGTGGACACTGATACT3'		
AP-07 / AP-08	Forward	5'CGTACTGTGGCTCAAAACTC3'	1400	58°C
	Reverse	5'ATTCGCACCGTTTAGCCCGT3'		

with each primer set based on 13 samples. Following the completion of the PCR reactions, the samples were subjected to electrophoresis in agarose gels, stained with ethidium bromide, and visualized under UV light, similar to the procedure used for DNA quality assessment.

3. Results

There is no existing chemical control which controls viral diseases therefore developing resistant cultivars against viral diseases in pepper is one of the most critical strategies for disease management. In advanced technology, marker-assisted selection (MAS) at the DNA level has emerged as a safer and more sustainable molecular method for resistance breeding. For MAS screening, high-quality nucleic acids are the first step toward success in molecular studies. Therefore, it is crucial in such studies, to obtain nucleic acids that are able to be used in a highly pure form both quickly and economically.

The selection of the most appropriate technique depends on several criteria, including target nucleic acid (DNA or RNA), source organism and type of plant material (leaf, seed, peel tissue, etc.), whether the leaf is young or mature, the timing of the study, the storage method, quantity, and location of the samples, as well as the desired outcomes (yield, purity, time required for purification, etc.). The applications following extraction also significantly impact the cost and success of the technique. For this purpose, three different methods, two different phenological stages, and two testing times were analyzed using two resistance markers in PCR studies.

When comparing the methods, the highest DNA concentration was obtained using the CTAB method yielding 391.23 ng μl^{-1}, followed by the Dellaporta and the commercial kit respectively. One of the key indicators affecting DNA quality is A260/A280 ratio which is used to assess nucleic acid purity. The absorbance ratio of approximately 1.8 indicates pure DNA, while a value around 2.0 suggests pure RNA. A 260/280 ratio greater than 1.8 indicates RNA contamination, while a 260/230 ratio below 1.8 suggests protein contamination. Any type of contamination may lead to several issues including poor outcomes or sample loss in molecular studies. Contamination also affects a shelf life of the sample causing quick degradation of nucleic acids (Yörek 2005). Spittle et al. (2010) reported that chemicals, purification method, plant materials and extraction environments cause contamination, thereby lowering the 260/280 ratio (Furda et al. 2014; Pierre et al. 2018). Considering all our general findings, it was determined that the commercial kit provided the most optimized DNA isolation method based on 260/280<1.8 and 260/230<1.8 ratios (Table 2). The highest DNA yield was achieved using the CTAB method from the first true leaf stage (Table 2).

As can be seen in Table 3, the commercial kit with an average of 144 ng μl^{-1} DNA obtained from the first true leaf, there was not only the highest quality but also the cleanest DNA extracted with absorbance A260/A280 1.860 and 260/230 1.981 ratios. Although, the highest DNA was obtained with an average of 325.42 ng μl^{-1} in the CTAB method, while it was desired to be 260/280>1.8, this ratio remained at 260/280 1.308 in the isolation made from fresh leaves. The lowest DNA with the dirtiest phenolic compounds and RNA were obtained from samples stored at -80°C with Dellaporta. The amount of DNA was high in the samples taken from the first true leaf in the Dellaporta and CTAB methods where DNA quality was high and ratios were close to optimum values. The amount and quality of DNA isolated from the 5th true old leaf and their storage at -80ºC decreased. All the obtained data was subjected to the T test (Table 3), there was no statistical difference between Dellaporta and CTAB methods in terms of DNA amounts, while there was a significant difference between the commercial kit and the other two extraction methods (Table 3). When the old leaves and the leaves stored at -80ºC were statistically examined, a significant difference was found between CTAB and Dellaporta methods (Table 3).

The optimization of DNA isolation was based on comparisons of concentration, purity, and band clarity. Additionally, the suitability of the extracted DNA for PCR applications was assessed using molecular markers linked to the *Tsw*, *L3*, and *L4* resistance genes. For PCR analyses, SCAC568 marker associated with the *Tsw* gene for TSWV resistance, and CR-20, CAMS451 markers associated with *L3* and *L4* genes for PMMoV resistance were utilized respectively. The PCR amplifications were performed under optimized conditions, and the presence or absence of resistance alleles in the genotypes was determined based on the resulting band patterns on agarose gels.

The results indicated that although all three methods successfully yielded DNA suitable for molecular studies, the modified CTAB method provided higher DNA concentrations and better-quality results compared to the others. The Dellaporta method yielded relatively lower concentrations but still produced DNA suitable for PCR. While the commercial kit offered convenience and consistent purity, it was found to be less cost-effective for large sample sizes (Martellossi et al. 2005).

Proteins absorb at 280 nm, the A260/A280 ratio is commonly used to assess nucleic acid purity. Pure DNA typically yields a ratio of approximately 1.8, whereas pure RNA gives a value around 2.0. The 260/280 ratio greater than 1.8 indicates RNA contamination, while a ratio lower than 1.8 at 260/230 suggests protein contamination (Shokere et al. 2009; Sedlackova et al. 2013). Any form of contamination can lead to numerous problems. In molecular studies, contamination may result in poor outcomes and even the loss of the sample. Furthermore, contamination negatively affects the storage stability of the sample, potentially causing a more rapid degradation of the nucleic acids obtained (Yörek 2005; Luciano et al. 2007; Visvikis et al. 1998). According to Spittle et al. (2010), Gallagher (2011) and Glasel (1995), chemicals used during extraction, the purification method, and the working environment or materials can all contribute to contamination, thereby decreasing the 260/230 ratio.

To assess the impact of DNA quantity and quality on molecular analyses, PCR assays were conducted using five commercial pepper cultivars that carry the *L4* and *Tsw* resistance

Table 2. Plant materials and DNA extraction methods are compared with absorbance ratios and provide the best DNA extraction methods

Methods		Plant Material								
		First real leaf			Old leaf			90 days stored leaf at -80°C		
DNA extraction method	No	Total DNA amount (ng µl^{-1})	260/230 Ratio	260/280 Ratio	Total DNA amount (ng µl^{-1})	260/230 Ratio	260/280 Ratio	Total DNA amount (ng µl^{-1})	260/230 Ratio	260/280 Ratio
Dellaporta	1	273.18	1.432	2.203	201.01	1.008	0.815	187.18	0.998	0.789
	2	247.23	1.682	1.987	113.32	0.948	0.997	99.14	0.902	0.824
	3	260.33	1.340	2.036	148.36	0.961	1.011	88.16	0.879	1.001
	4	261.28	1.548	2.502	217.28	1.089	0.903	151.99	0.856	0.789
	5	305.56	1.246	1.525	120.54	1.023	1.107	108.09	0.826	0.698
	6	236.58	1.585	1.966	139.78	0.893	0.963	90.25	0.905	0.891
Mean		**264,01**	**1.472**	**2.037**	**156.15**	**0.897**	**0.966**	**120.80**	**0.909**	**0.832**
CTAB	1	260.06	1.514	2.098	113.31	1.110	0.996	93.31	0.986	0.891
	2	307.36	0.958	1.628	120.11	0.893	1.004	80.56	0.754	0.904
	3	391.23	1.457	2.190	186.34	0.991	1.001	93.65	0.587	0.801
	4	248.60	1.482	1.963	119.05	1.594	1.028	102.45	0.759	0.831
	5	344.43	0.859	1.588	105.26	0.981	1.208	78.05	0.802	1.154
	6	400.85	1.580	1.869	213.62	0.944	0.919	102.30	0.659	0.958
Mean		**325.42**	**1.308**	**1.839**	**142.95**	**1.085**	**1.026**	**91.72**	**0.757**	**0.923**
Commercial Kit	1	121.12	1.845	2.090	98.45	1.113	1.112	76.42	1.001	1.003
	2	118.34	1.861	1.986	99.68	1.216	1.201	69.69	0.989	1.102
	3	178.87	1.885	2.032	100.24	1.227	1.138	70.12	1.015	1.003
	4	150.34	1.933	1.989	86.00	1.025	1.094	75.20	1.596	1.601
	5	130.25	1.650	1.799	102.33	1.079	1.064	80.03	1.036	1.102
	6	169.02	1.991	1.981	98.37	1.198	1.289	83.26	0.936	1.025
Mean		**144.65**	**1.981**	**1.860**	**97.51**	**1.143**	**1.145**	**75.78**	**1.096**	**1.156**

Table 3. Values of each DNA extraction method analyzed in T statistical test

Plant Tissue	Measurement	DNA Extraction Method	Compared Extraction Method	Statistical Deviation[¥]
First real leaf	Total DNA amount	Dellaporta	CTAB	NS
		Dellaporta	KIT	**
		CTAB	KIT	**

*Significant at the 0.05 probability level, **Significant at the 0.01 probability level, [¥]Differences between means of methods and DNA values were tested by *t* test, NS: none significant.

genes, along with the susceptible commercial variety which lacks both L4 and *Tsw* resistant genes.

PCR was performed using DNA extracted by three different methods from three different sample types, targeting the *Tsw* CAPS marker for resistance to Tomato Spotted Wilt Virus (TSWV) and the Ap7/Ap8 marker used for resistance to Pepper Mild Mottle Virus (PMMoV).

Despite reduced DNA quality and quantity, the L4 primer was successfully amplified in all samples. However, the Tsw CAPS marker for TSWV resistance only produced reliable amplification in the samples obtained from the first true leaves across all nine combinations. In contrast, DNA extracted from the 5 true leaf and stored at –80°C, using the CTAB, Dellaporta, and a commercial kit extraction method resulted in low products in PCR reactions where the Tsw CAPS marker did not perform as effectively. In this study, PCR was conducted on five resistant and susceptible commercial F1 hybrid pepper genotypes using DNA extracted to identify the presence of 2 genes, the *L4* gene confers resistance to Tobacco Mosaic Virus (TMV), Pepper Mild Mottle Virus (PMMoV), and the *Tsw* gene provides resistance to Tomato Spotted Wilt Virus (TSWV). Different DNA extraction methods with the resistance genes were evaluated for such devastating diseases caused by *Tobamoviruses* and TSWV in pepper. Upon examination of the PCR results, it was found that all three DNA isolation methods performed well when DNA was extracted from the first true leaves. As shown in Figure 1A commercial genotype lacked the *Tsw* gene, while the other five genotypes carried this resistance gene. After digestion with the *XbaI* enzyme, it was confirmed that these five genotypes were resistant (Figure 1B). However, when DNA was extracted from older leaves or from samples stored at -80°C, the *Tsw* gene failed to amplify reliably across all three methods. This suggests that DNA from the first true leaf must be used when analyzing the *Tsw* gene in pepper. Otherwise, the other commercial genotypes were genetically resistant but the resistant bands did not appear, while susceptible genotype bands were visible (Figure 1C). Despite the high DNA quantity, the presence of phenolic compounds negatively affected quality with 260/280 ratios ranging from 0.832 to 1.156, and 260/230 ratios between 0.757 and 1.085 values that deviate significantly from the desired thresholds. While a 260/280 ratio>1.8 indicates RNA contamination, a 260/230 ratio<1.8 is generally acceptable but the values from older or frozen leaves were far from ideal and the ratio was negatively affected (Figure 1D) in the PCR outcomes (Nadeem et al. 2017; Mitsouras and Faulhaber 2009; Bozkaya 2012; Şimşek et al. 2008).

Regarding PMMoV resistance, the *L4* gene was successfully detected using AP7-AP8 primers in PCR conducted with DNA extracted using all three methods at different time points. These results highlight the need to optimize conditions for specific gene markers, these results were consistent with results in Nadeem et al. (2017). In conclusion, when working with the *Tsw* gene in pepper, it is essential to use DNA extracted from the first true leaf under optimal conditions. For the *L4* gene associated with PMMoV resistance, the AP-7 and AP-8 primers amplified product in all tested conditions.

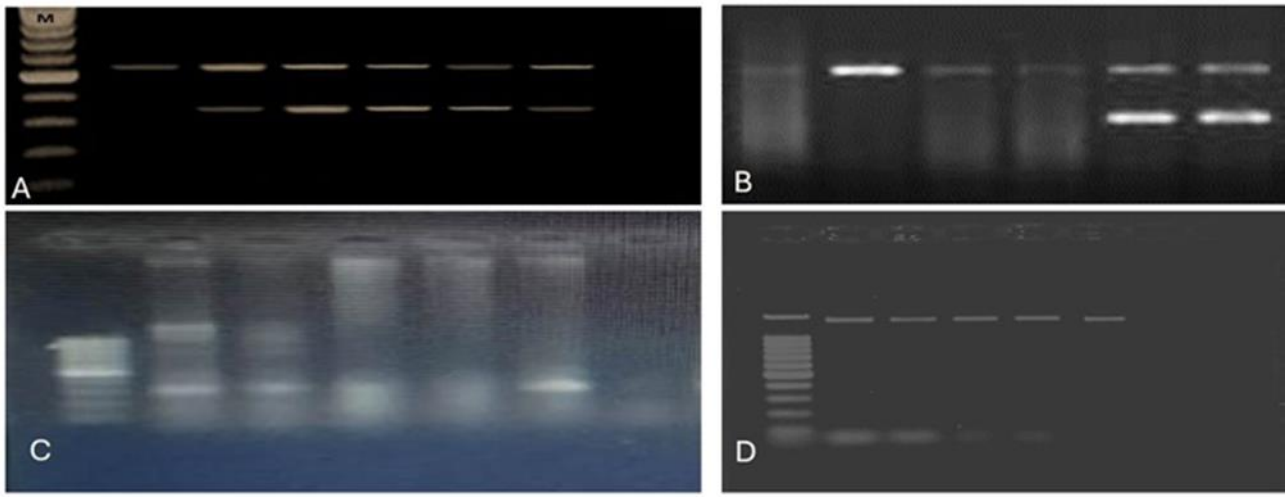

Figure 1. The gel visualizations of *Tsw* and *Ap7*/*Ap8* primers are used to asses DNA qualities obtained with different extraction methods in PCR. A) The CAPS primers have amplified products on agarose gel where first real leaf was used to extract DNA with commercial kit, B) An agarose gel view with old leaf DNA samples were extracted with Dellaporta, C) The AP primers have amplified products on agarose gel where 90 days old leaf samples wereused to extract DNA using CTAB, and D) The AP primers have amplified products on agarose gel where 90 days old leaf samples were used to extract DNA using commercial kit method.

Although potassium acetate yielded lower DNA concentrations than phenol-chloroform for protein removal during DNA isolation (Bozkaya 2012), it still provided DNA of sufficient quantity and quality, hence, the potassium acetate may be preferred over phenol-chloroform which poses health and environmental risks (Bozkaya 2012). Furthermore, careful handling and preservation of plant tissues positively influence the quality of the isolated DNA.

4. Discussion and Conclusion

For the successful isolation of total nucleic acids, DNA should be free from both organic and inorganic contaminants. As the isolation protocol progresses and the sample becomes purer, more delicate handling is required. For instance, the tubes should not be vortexed or exposed to high RPM centrifugation. DNA can be shared due to excessive agitation or damage from impacts and may degrade if exposed to high temperatures for extended periods.

If the DNA is not handled cleanly, PCR analyzes may be inhibited, resulting in failed amplification or low-quality band patterns. Another key factor affecting DNA quality is the developmental stage of the plant at the time of sampling. In breeding studies, especially for marker analysis, using the first true leaf that emerges after the cotyledon provides more reliable results. Whenever possible, DNA isolation should be performed immediately after sampling from fresh tissue. Even when young leaves are collected from older plants, DNA quality tends to decline with plant age.

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