EMPREGO DO MARCADOR SCAR NA DISCRIMINAÇÃO DE CULTIVARES DE VINCA (*Catharanthus roseus* (L.) G.Don)[1]

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RESUMO - A técnica "Sequence-Characterized Amplified Region" (SCAR) pode ser ferramenta útil na análise da pureza genética e discriminação de cultivares, especialmente quando se trabalha com espécies em que os resultados obtidos com RAPD não foram consistentes. Neste estudo, bandas polimórficas RAPD geradas a partir de DNA de tecido foliar, foram clonadas e seqüenciadas e pares de "primes" SCAR foram então desenvolvidos para utilização na reação de PCR. Os padrões de bandeamento SCAR obtidos a partir de DNA extraído de sementes de vinca (*Catharanthus roseus* (L.) G.Don) foram consistentes e repetitivos, gerando resultados confiáveis para teste de pureza genética e discriminação de cultivares. Considerando que a probabilidade de sucesso na obtenção de "primers" SCAR a partir de bandas polimórficas de RAPD é de aproximadamente 50%, um grande número de bandas polimórficas RAPD é necessário para desenvolver quantidade de "primers" SCAR suficiente para discriminação de cultivares de vinca. Além deste aspecto, a eficiência da técnica SCAR foi menor quando se utilizou DNA extraído de sementes do que quando se utilizou DNA extraído de folhas. Este resultado foi devido à qualidade inferior do DNA extraído das sementes. O presente estudo mostrou que a técnica SCAR é simples e rápida e de custo relativamente baixo após o desenvolvimento dos "primers". Esta técnica permite o uso de DNA extraído de sementes secas, o que é um fator fundamental no programa de avaliação da pureza genética ou varietal de lote de sementes.

Termos para indexação: vinca, sementes, discriminação de variedades, marcadores moleculares, SCAR.

SEQUENCE-CHARACTERIZED AMPLIFIED REGION (SCAR) TECHNIQUE USED FOR VARIETY DISCRIMINATIION IN VINCA (*Catharanthus roseus* (L.) G.Don)[1]

ABSTRACT - Sequence-Characterized Amplified Region (SCAR) appears as a useful technique for genetic purity testing and variety discrimination, applicable to species in which some other techniques have failed. In particular, this technique is very attractive with species in which RAPD results were not consistent. The RAPD polymorphic bands were cloned, sequenced and from the sequence information, primers pairs for normal PCR were developed. Since the probability of obtaining successful SCAR primers from RAPD polymorphic bands was about 50%, a larger number of RAPD polymorphic bands are needed to develop sufficient SCAR primers for varietal discrimination in vinca. In addition, the efficiency of the SCAR technique is strongly affected by the quality of DNA extracted from seeds. The SCAR banding patterns obtained from vinca seed were consistent and repeatable making the results reliable for genetic purity testing and variety discrimination. The SCAR technique is simple, fast, relatively inexpensive and allows the use of DNA extracted from dry seeds, which is very important in a seed-quality evaluating program

Index terms: vinca, seed, variety discrimination, molecular markers, SCAR.

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INTRODUCTION

New varieties are continually being developed and released, especially among those species commercially important. This trend is clearly observed in the development of ornamental plant varieties as an evident consequence that ornamentals are becoming increasingly popular in the world. As a result of the large number of varieties and the broadening use of biotechnology, varieties are becoming very similar, sometimes differing only in one single gene. Therefore, testing for genetic purity and variety discrimination became a more difficult and complex task. Traditionally, genetic purity testing has been accomplished using morphological traits expressed by the seed, seedling and/or mature plant. Morphology, however, cannot provide information on the genetic attributes related to grain quality or stress resistance incorporated into contemporary varieties. In addition, this seed purity analysis using morphological traits usually is labor intensive and time consuming. For this reason, faster and more reliable techniques are being developed and refined in order to achieve this objective.

Electrophoresis of seed and seedling proteins/isoenzymes is one of the relatively new approach to genetic purity testing. Although this technique is useful in some situations, it still fails to differentiate a number of varieties in some crops, particularly in vegetables and flowers which lack sufficient protein/isoenzyme heterogeneity among parental lines (Della-Vecchia at al., 1998). More recently, DNA-based technologies based on the Polymerase Chain Reaction (PCR) technique have been developed and used successfully for variety discrimination. This technique is an ingenious and powerful tool capable to amplify a single DNA molecule, consequently very promising for discrimination of close related varieties. PCR associated with a genetic assay called Random Amplified Polymorphic DNA (Williams et al., 1990; Welsh & McClelland, 1990) has led to a new generation of molecular marker-based genetic purity determinations. The amount of DNA produced can be visualized as discrete bands in an agarose gel following electrophoresis. However, RAPD is considered a non-reproducible technique by some researchers, consequently producing results non repeatable among different laboratories (Riedy et al.1992; Heun & Helentjaris, 1993; Smith & Register, 1998). This characteristic can lead to erroneous conclusions concerning genetic purity. The low stringency requirements consider for some as the great advantage of RAPD, can cause irreproducible and unexpected results in some cases (Riedy at al., 1992; Heun & Helentjaris, 1993), especially when not using the most appropriated tissue as DNA source. As a consequence of low complementarity between the primer and target DNA sequence, the test is difficult to standardize and obtain repeatable results, even in the same laboratory. This may be the reason for the failure of RAPDs in some studies to generate consistent and repeatable data, especially when using DNA extracted from seed tissue.

The objectives of this research were to develop longer and more stringent primers from polymorphic RAPD fragments and evaluate the consistency and applicability of this technique for variety discrimination.

MATERIALS AND METHODS

This work was carried out in the USDA Laboratory located in the Food, Agricultural and Biological Engineering Department at the Ohio State University, USA, during the period of 11/21/98 to 06/15/99.

Plant material - seeds of 10 varieties of vinca, (Vinca Peppermint, Vinca Strawberry, Vinca Blue Pearl, Vinca Blush Cooler, Vinca Rose Cooler, Vinca Raspberry, Vinca Grape Cooler, Vinca Icy Pink, Vinca Orchid Cooler, Vinca Coconut Cooler and Vinca Pink White Eye) were obtained from Ball Seed Company, West Chicago, IL.

DNA extraction, quantification and dilution - in most procedures, DNA from seeds were extracted following the extraction protocol described by McDonald at al. (1994). For comparison, DNA from seeds was also extracted by the proteinase K protocol described by Zhang at al. (1996). Twenty seeds of each variety were sowed in pots and grown for 20 days. Leaves from each plant in the pot were evenly harvested, cut into small pieces, uniformly mixed and then four grams from each variety were used. Thereafter, DNA extracted following the CTAB procedure described by Saghai-Maroof et al. (1984). DNA quantification was performed in a BECKMAN, DU - 50 spectrophotometer using optical density (OD) readings of 260 and 280nm. The first value refers to the DNA concentration while the ratio OD_{260}/OD_{280} assessed the purity of the DNA sample. The concentration ranged from $400ng.\mu l^{-1}$ to $800ng.\mu l^{-1}$, while the ratio OD_{260}/OD_{280} ranged from 2.0 to 2.3. The final DNA concentration of all 10 samples was adjusted to $\sim 10ng.\mu l^{-1}$ by diluting the concentrated extraction solution in sterile double distilled water; **DNA amplification (RAPD) -** after dilution to $\sim 10ng.\mu l^{-1}$, 2µl of each DNA sample were added to a reaction tube containing 24µl of PCR solution [20mM Tris-HCl, pH 8.4, 50mM KCl, 3mM $MgCl_2$, 200µM of each dATP, dCTP, dGTP, dTTP, 1.5

units *Taq* DNA Polymerase (Perkin Elmer, Foster City, CA) and 0.4μM primer]. Four 10-mer primers (Ransom Hill Bioscience, Inc., Ramona, CA) useful for DNA amplification in Vinca studies were used: 262 (CGCCCCCAGT), 244 (CAGCCAACCGG), 07 (CGGCCACTGT) and 01 (CGGCCCCTGT). The reaction tubes with the complete DNA amplification mixture, and a control containing all the reactants excluding extracted DNA, received a drop of mineral oil to minimize evaporation. Subsequently, the tubes were vortexed, centrifuged and placed in a DNA thermal cycler (PTC - 100 MJ Research, Inc.) programmed for the following temperature cycles repeated 40 times: 94°C, min; 40°C, one min.; 72°C, two min. An initial temperature of 94°C for three min. and a final temperature of 72°C for 10 min. were adjusted for DNA denaturation and extension, respectively. After approximately four hours, the tubes were removed from the thermocycler, centrifuged at 10,144 x g for 30 sec., avoiding any loss of material; **electrophoresis** - was conducted according to McDonald et al. (1994), except that 12.5ml of the amplified sample were loaded onto the 1.5% agarose gel in order to visualize the polymorphic bands; **extraction from the gel and purification** - the polymorphic bands were viewed under a transluminator Model Foto/PrepI (Fotodyne, Hartland, WI) and then excised from the agarose gel with a clean, sterile scalpel. The gel slice was minimized up to approximately 100 mg by removing extra agarose. DNA extraction and purification was performed using the QIAquick Gel extraction kit; **DNA quantification and rePCR** - after extraction and purification, the DNA fragments were quantified in a spectrophotometer BECKMAN, DU - 50 using optical density (OD) readings of 260 and 280nm. The first value refers to the DNA concentration while the ratio OD_{260}/OD_{280} assessed the purity of the DNA sample. rePCR was performed as described for DNA amplification, except that 10ng of purified DNA fragment were added into a PCR reaction as the template; **ligation reaction** - after rePCR, 10ng of fresh purified PCR product were cloned into a vector**,** pCR™ 2.1**,** from **TA cloning^R Kit.** The ligation reaction (10μl) contained: 5μl of sterile water; 1μl of 10X ligation Buffer; 2μl of pCR**^R** vector (25ng μl^{-1}); 1μl of fresh PCR product (~10ng) and 1μl of T4 DNA Ligase. Incubate ligation reactions at 14°C for at least four hours (preferably overnight). After that, ligation reactions were briefly centrifuged and placed on ice; **transformation** - transformation was achieved by heat shock technique using competent cells of bacteria *Enchirichia coli*. One hundred μl of bacteria were removed from the - 80°C freezer and kept on ice. Following that, 10μl of ligation reaction were added to the bacteria volume and maintained on ice for 45 minutes. Immediately, the total volume containing bacteria and ligation reaction was placed at 37°C waterbath for five minutes. Following this, 1μl of SOB Medium was added to the transformed bacteria and transferred to an incubator at 37°C with gently shaking for 45 minutes. Thereafter, 100μl of medium having bacteria along with vector and the inserted DNA fragment were loaded in the LB plates containing ampicillin (50mg.μl^{-1}), IPTG (70mg.μl^{-1}) and X-GAL (40mg.μl^{-1}) and incubated at 37°C overnight; **single colony selection** - bearing the vector could grow in the medium containing ampicillin since the gene encoding for resistance was present. Those bacteria bearing the vector plus the inserted DNA fragment grew white, while those without the fragment turned to blue. Five individual white colonies, for each specific DNA fragment, were selected for PCR. The PCR reaction was performed as described for DNA amplification using the M13 forward (CTG GCC GTC GTT TTA C) and reverse (CAG GAA ACA GCT ATG AC) primers. The following temperature program was repeated 30 cycles: 94°C, three min.; 50°C, one min.; 72°C, 40 sec. An initial temperature of 94°C for three min. and a final temperature of 72°C for 10 min. were added for DNA denaturation and extension, respectively. The concentration of primers was the same as for RAPD (0.4μM). DNA template was obtained from the single white colony by touching it with a pipette tip and place into the PCR reaction microtube with a gentle stirring. After PCR, electrophoresis was conducted according to McDonald et al. (1994), in order to visualize those specific fragments. Based upon the bands visualized in the gel the single colonies bearing polymorphic fragment were selected; **plasmid extraction and quantification** - the selected colony from each fragment was touched with a pipette tip and introduced into a 15μl falcon tube containing 5μl of Lurial-Bertani plus Ampicillin medium to grow overnight at 37°C with gentle shaking. In the following day the tubes were centrifuge at 2500 Relative Centrifugal Force (RCF) for five min. in order to obtain a pellet. The extraction itself was performed according to QIAprep Spin Miniprep kit. The plasmid concentration was quantified in a spectrophotometer as described previously; **DNA sequencing** - the PCR samples preparation, thermocycling conditions, purification of PCR products, speed vacuum to dryness and sequencing followed the Big Dye Terminator kit (ABI PRISM). The clones were sequenced by the dideoxy nucleotide terminator method using the ABI Prism 310 (PE Applied Biosystems, Foster City, CA). The cycle Sequencing reaction was performed in a Perking

Elmer Gene Amp PCR system 9600 using the Big Dye™ sequencing Kit and M13 forward and reverse primer following the manufacture protocol; **scar primers development** - the sequenced DNA, reversed and forwarded, was matched with the known vector sequence in order to recognize the precise starting point of the polymorphic vinca fragment. It started with the RAPD primer sequence used to amplify the specific fragment, and had more approximately 200 unambiguously nucleotides. Afterwards, some other nucleotides were added to the original RAPD primer sequence to give rise a longer primer. The quality of those longer primers was analyzed in the internet by checking the following home pages: http://www.idtdna.com/index.html and http://www.williamstone.com/primers/calculator/). The CG content, melting temperature, hairpin and self-dimmer formation were evaluated; **PCR reaction and electrophoresis** - PCR reactions were performed as described for single colony selection, except that the annealing temperature varied according to the melting temperature for each pair of primer (forward and reverse), and the used template was DNA extracted from seeds. Electrophoresis was conducted according to McDonald et al. (1994), in which samples were loaded onto the 1.5% agarose gel and bands were viewed under a Transluminator Model Foto/PrepI (Fotodyne, Hartland, WI).

RESULTS AND DISCUSSION

The four RAPD primers that gave the best results for vinca variety discrimination (Menezes at al. 2001)[6] were used to generate polymorphic bands (Table 1). It is possible to check the polymorphic bands generated when using only one RAPD primer (Figure 1). Eight RAPD polymorphic fragments were generated and later developed into SCAR primers (Table 2). Almost all the new designed longer primers incorporated the original RAPD primer, plus some other sequenced nucleotides (Table 2).

According to the literature, using longer primers the annealing temperature and the annealing site during PCR reaction become more stringent (Innis et al., 1990), consequently more reproducible, overcoming the RAPD technique main limitation (Smith & Register, 1998).

Four longer developed primers were able to generate polymorphic bands when using DNA extracted from leaves, while only three succeed with DNA from seeds.

As shown on Table 3 and Figure 2, it was possible to identify the following varieties: Cooler Vinca Raspberry, Vinca Orchid Cooler and Vinca Coconut. The other seven varieties were discriminated into three different groups. In order to individualize varieties into the same group, it is necessary to develop more SCAR primers and make sure they will proceed discriminative when applied to DNA from seeds.

It was observed that selected polymorphic DNA fragments generated using RAPD technique were

TABELA 1. Sequence of RAPD primers that generated polymorphisms among ten vinca varieties

RAPD primers	Sequence of RAPD primers
P - 262	CGC CCC CAG T
P - 244	CAG CCA ACC G
P - 07	CGG CCA CTG T
P - 01	CGG CCC CTG T

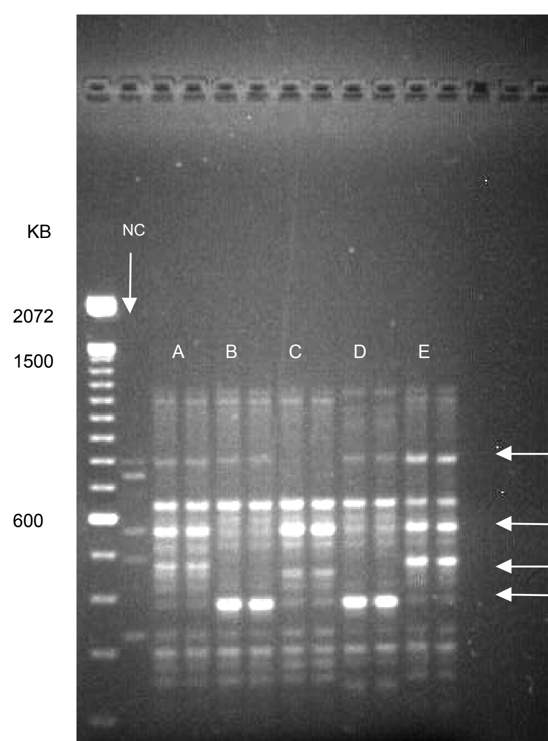

FIG. 1. Repeatability of RAPD bands in the same variety of vinca, using DNA extracted from leaves and loaded into the wells in pairs. Polymorphic RAPD bands generated by using primer 07 among five vinca varieties.

A - Vinca "Peppermint"; **B** - vinca "Blue Pearl"; **C** - vinca "Blush Cooler"; **D** - vinca "Rose Cooler"; **E** - vinca "Raspberry".

NC - negative control. The arrows indicate the polymorphisms among different varieties; KB - hilo bases (thousand pars of nitrogen basis).

TABELA 2. Sequence of SCAR primers developed from RAPD polymorphic fragments.

Identif. of fragment	Primer	Sequence of scar primers
250 BP	Forward	5'- CGG CCC CTG TAC CTG CTA C -3'
250 BP	Reverse	5'- CCC CTG TGG CGA TAA TTT CTT -3'
300 BP	Forward	5'- CGG CCC CTG TTA AGT AGT - 3'
300 BP	Reverse	5'- CCT GTT GGG ATG TAC TAT TAA A -3'
400 BP	Forward **	5'- CTA TTG CGG GCC TTG TTC -3'
400 BP	Reverse **	5'- CAC TGT TCA GGC AGG GG -3'
600 BP	Forward **	5'- CGC CCC CAG TAG AGG TTC -3'
600 BP	Reverse **	5'- CGC CCC CAG TTC AAA GAT -3'
700 BP	Forward	5'- CGC CCC CAG TTA GAG GAA -3'
700 BP	Reverse	5'- CGC CCC CAG TCT ACT ATG AT -3'
800 BP	Forward	5'- CGG CCA CTG TTC CTT AAT ATG -3'
800 BP	Reverse	5'- CGG CCA CTG TGG CAG AT -3'
850 BP	Forward **	5'- CAG CCA ACC GTT GGA TAT AT -3'
850 BP	Reverse **	5'- CAG CCA ACC GAG GCG -3'
900 BP	Forward *	5'- CCA CTG TCA CCC ACC TCA C -3'
900 BP	Reverse *	5'- CGG CCA CTG TAA CAG GGT -3'

BP - base pairs.
** amplification of DNA extracted from leaves and seeds; * amplification of DNA extracted from leaves.
Bolded letters - Original sequence of RAPD primer.

TABLE 3. Identification of ten vinca varieties using DNA extracted from seeds, amplified using four pairs of primer SCAR (400, 600, 700 and 850 bp).

Varieties	Group	Polymorphic bands			
		400	600	700	850
Vinca Pepermint	1	–	+	+	-
Vinca blue Pearl	1	–	+	+	–
Vinca Blush Cooler	1	–	+	+	–
Vinca Strawberry	2	+	–	–	–
Vinca Rose Cooler	2	+	–	–	–
Vinca Grape Cooler	3	–	–	–	–
Vinca Pink White Eye	3	–	–	–	–
Vinca Raspberry	5	+	–	–	+
Vinca Orchid Cooler	6	–	+	–	–
Vinca Coconut Cooler	7	–	–	+	–

(+) - Presence of the polymorphic band.
(-) - Absence of the polymorphic band .

distinguishable when extracted from the gel (Figure 1). However, some of the SCAR primers developed from those fragments did not generate polymorphic bands. They amplified that specific fragment in all varieties, hence not useful for discrimination. As some researches showed RAPD technique not to be repeatable (Riedy et al., 1992; Heun & Helentjaris, 1993), it is possible that some of the extracted RAPD polymorphic fragments were not really polymorphic. Somehow, the RAPD primers used to generate polymorphic fragments might have annealed improperly in some varieties but not in others, as a consequence of low stringent condition. As a result, polymorphic bands were observed in the gel even though they were originated from mismatch. Then, those fragments generated by mismatching were extracted from the gel, sequenced and SCAR primers developed. When the developed primers were used in a more stringent condition, i.e., longer annealing target and higher annealing temperature, the previous inaccurate mismatches did not occur, hence polymorphisms were not verified. It is in accordance with the literature that longer annealing site and higher annealing temperature avoid mismatching (Innis et al., 1990; Ferreira & Grattapaglia, 1996).

One of the designed primer did amplify polymorphic bands when the original RAPD sequence was incorporated, but did not when the original 10-mer primer was not incorporated (Table 2). On that account, we attribute that the alteration generating polymorphism among varieties occurred on the template site where that specific RAPD primer had annealed. Hence, when the designed longer primer did not include the original 10-mer sequence all varieties were amplified (primer 600 bp). It is in accordance with Ferreira & Grattapaglia (1996), in which any modification in the annealing site can compromise further steps of amplification.

Another longer primer was able to generate polymorphic band when using DNA extracted from leaves but not when using DNA extracted from seeds (Figure 3). These results suggest difference in DNA quality based upon the DNA source. It can be confirmed by comparing the quality of DNA sources, which was accomplished by reading the ratio OD_{260}/OD_{280} and running a gel loaded with genomic DNA (Figure 4). The ratio OD_{260}/OD_{280} ranged from 2.0 to 2.3 in DNA extracted from leaves and 1.2 to 1.5 in DNA from seeds. As a consequence, any contaminate present in the DNA template from seeds might have impaired the primer to anneal on the

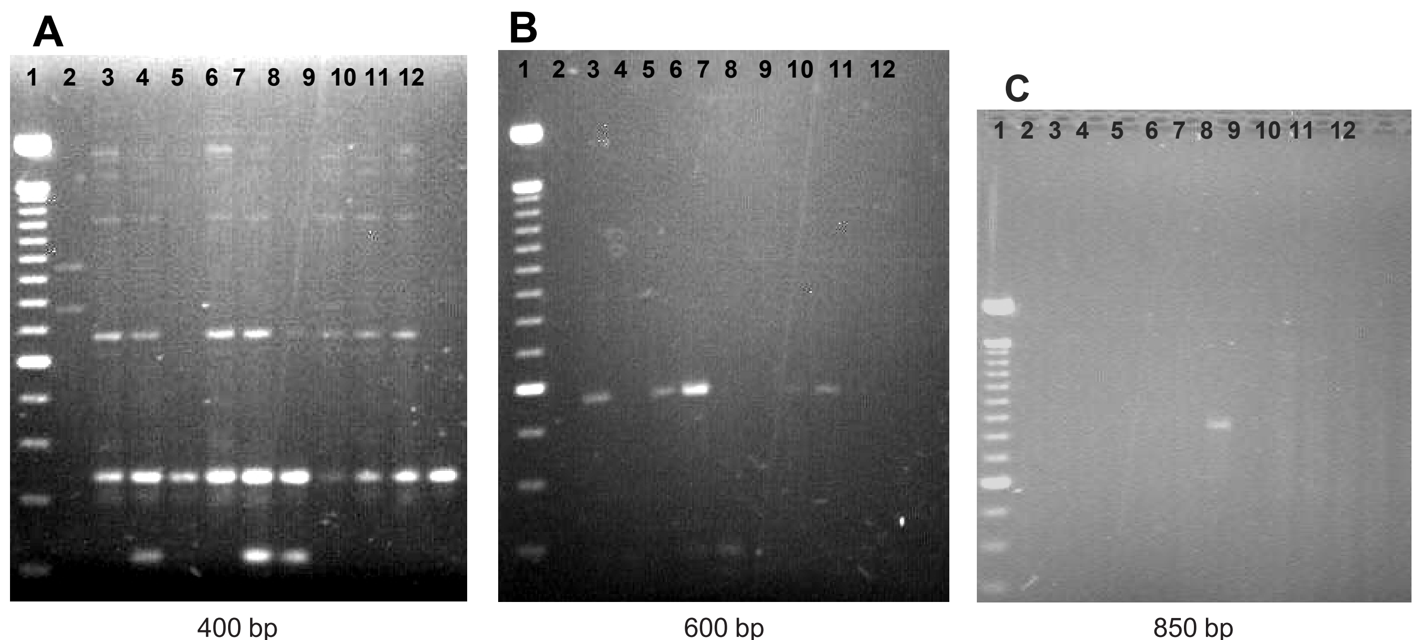

FIG. 2. SCAR polymophisms among ten vinca varieties generated by the amplification of DNA extracted from 100 mg of seeds of each variety.

A - "Primer" 400bp; **B** - "primer" 600bp; **C** - "primer" 850bp. **1** - Size marker; **2** - negative control; **3** - "Peppermint"; **4** - "Strawberry"; **5** - "Blue Pearl"; **6** - "Blush Cooler"; **7** - "Rose Cooler"; **8** - "Raspberry"; **9** - "Grape Cooler"; **10** - "Orchid Cooler"; **11** - "Coconut Cooler"; **12** - "Pink White Eye".

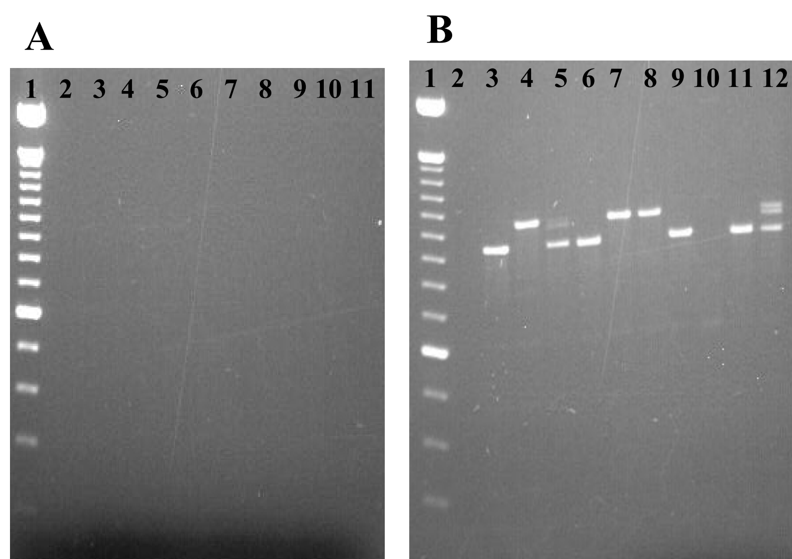

FIG. 3. Absence of amplified bands (A) with primer 950bp using DNA extracted from dry seeds of vinca and presence of amplified bands (B) using DNA extracted from leaf tissue.

1 - Size marker; **2** - negative control; **3** - "Peppermint"; **4** - "Strawberry"; **5** - "Blue Pearl"; **6** - "Blush Cooler"; **7** - "Rose Cooler"; **8** - "Raspberry"; **9** - "Grape Cooler"; **10** - "Orchid Cooler"; **11** - "Coconut Cooler" e **12** - "Pink White Eye".

target site and properly amplify the fragment. This is in accordance with Ferreira & Grattapaglia (1996), in which cleaner extracted DNA produced more reliable results. According to Marcos-Filho & McDonald (1998) the majority of DNA extracted from soybean seeds generated good-quality DNA within the ratio OD_{260}/OD_{280} ranging from 1.6 to 1.8, hence achieving consistent and repeatable results. High quality DNA in vinca seeds was not achieved using neither quick buffer procedure nor adding proteinase K (Life Technologies, Gaithersburg, MD) as recommended for soybean (Zhang et al., 1996; Marcos-Filho & McDonald, 1998). It is possible that proteinase K played an important role in improving DNA quality in soybean seeds regarding that the amount of protein in soybean seeds is much higher than in vinca seeds. However, DNA extracted from vinca leaves showed very high quality in both evaluation, i.e., ratio OD_{260}/OD_{280} and running a gel with genomic DNA (Figure 4).

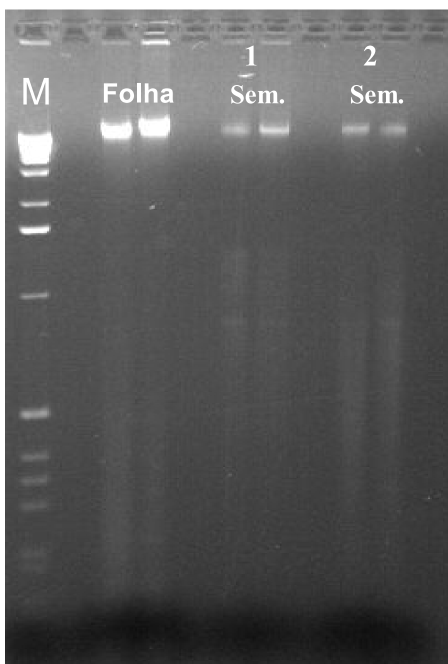

FIG. 4. Quality comparison between DNA extracted from leaf tissue and from dry seeds of vinca.
1 -"proteinase K" method and **2** - quick method.
Two application of 1µg from each extraction into every well.

CONCLUSION

- The SCAR technique is reliable and consistent for genetic purity testing and variety discrimination in vinca since it is a DNA-based technique;
- SCAR technique has the advantage of use more stringent conditions than RAPD, enabling to overcome the RPAD reproducibility limitation;
- it is necessary to perform some adjustment in the dry-seed DNA extraction procedure in order to achieve higher DNA quality;
- the technique might be applicable to vinca seeds as well as many other species in which molecular techniques still fail in testing genetic purity and variety discrimination.

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