



Inter-simple Sequence Repeat (ISSR) Analysis of Somaclonal Variation in *Artemisia amygdalina* Decne. plantlets Regenerated from nodal explants

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ABSTRACT

In this study *Artemisia amygdalina* - a critically endangered medicinal plant endemic to Kashmir Himalayas was grown under *in vitro* conditions. ISSR markers were used to study the somaclonal variation at the genetic level. Two ISSR-primers (CAC)₃ GC and (ACTG)₅ were used, two additional DNA bands (0.15kb and 0.2kb) were observed respectively in tissue cultured *Artemisia amygdalina*. Direct regeneration was seen from nodal explants on MS + BAP (10µM) + (NAA (10µM). The results depict that direct regenerants were actually the somaclonal variants but not true to type as was expected.

Key Words: *Artemisia amygdalina*; ISSR; somaclonal variation; endangered

INTRODUCTION

Somaclonal variation describes epigenetic and genetic changes in plants that become apparent either during or after *in vitro* culture of plant cells, callus or organs (Larkin & Scowcroft 1981). This variation may be caused through pre-existing genetic variation occurred in the explant and the variation induced by the *in vitro* conditions (Skirvin, *et al.* 1994). This variation is manifested in the form of DNA methylations, chromosome rearrangements and point mutations (Phillips *et al.* 1994). Though, whether somaclonal variation may imply an advantage as a source of variability for new lines, or a disadvantage for the propagation of an elite cultivar, it is important to achieve a rapid and easy method to assess the genetic stability of the propagated plants at the earliest stage of plant growth.

Genetic variation analyses can be observed from morphological characters and other markers as protein or deoxyribonucleic acid (DNA)-based markers. However, the morphological characters evaluation requires the plants to grow to full maturity prior to identification. Analyses of secondary metabolites and isozyme patterns have

also been used, but they are limited in their sensitivity (Morell, *et al.* 1995). The progress made in DNA marker technology has been tremendous and exciting. ISSR markers are simple and faster, needs only little amount of DNA and no need of radioactivity tests. These markers have been successfully used for the detection of somaclonal variation in various micropropagated plants (Carvalho *et al.* 2004; Martins *et al.* 2004; Ramage *et al.* 2004; Modgil *et al.* 2005). ISSR is highly discriminative, reliable and cost-effective (Pradeep, *et al.* 2002; Mehrotra *et al.* 2012).

Artemisia amygdalina Decne. which is a critically endangered endemic species of Kashmir valley was cultured for the conservation of its germplasm. The *in vitro* grown plants show modifications from the wild species which can be essential, as the plants can show increased production of secondary metabolites useful in medicine. Since the *Artemisia* has high medicinal values, it has necessitated using molecular markers to find differences at the gene level. This genetic variation can serve the best tool to search for the medicinally important species (Avisé 1994).

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MATERIAL AND METHODS

Extraction and analysis of DNA

To isolate DNA the CTAB-DNA isolation method was followed with slight modifications (Tewary and Suryanarayana 2007). DNA was isolated from the leaves of *in vitro* grown *Artemisia amygdalina* and that growing in natural conditions. Isolated DNA was dissolved in 50 μ l Tris-EDTA buffer and stored at 4°C. Genomic DNA was run on 0.8% agarose gel. DNA sample (2 μ l) was mixed with 1 μ l of 1X DNA loading dye (4.16 mg bromophenol blue, 4.16 mg xylene cyanol and 0.66g sucrose /ml H₂O). Gel was visualized by a Gel doc system (Alpha imagerTM 2200, Alpha Innotech Corporation) under UV light and picture was captured by using CCD camera system

ISSR analysis

ISSR-PCR reaction was performed in 25 μ l reaction volume containing 50ng genomic DNA, 0.3 μ l Taq DNA polymerase, 1x-10x PCR buffer, 2mM MgCl₂, 100 mM dNTPs and 5pmol ISSR primers. Amplification was carried out using T professional basic thermocycler (biometra) for 30 cycles programmed as follows:

Initial denaturation (one cycle) at 95°C for 5 min. followed by 35 cycles: as follows: denaturation at 94°C for 45 sec, annealing at 48°C for ISSR1 and 41°C for ISSR2 for 45 sec, elongation at 72°C for 1 min; final extension at 72°C for 7 min.

Primers used: ISSR1-(CAC)₃GC; ISSR2-(ACTG)₅

Gel Electrophoresis

Amplification mixture was mixed with 2 μ l of the gel loading dye {Sucrose 40% and Bromophenol Blue 0.25% (w/v)} and electrophoresed on 2.5% agarose gel stained with ethidium bromide along with 50ng of 50bp ladder (NORGEN Biotic Corporation) for 1hr at constant 100 volts. The gels were pictured in Gel-Doc system (Alpha imagerTM 2200, Alpha Innotech Corporation).

RESULTS

In PCR-ISSR analysis of *Artemisia* species using the primer 5' CAC CAC CAC GC 3' similar kind of band pattern was observed in all the four *Artemisia* species except for the minor variation in the pattern of the tissue cultured species of *Artemisia amygdalina* (Lane 3) and wild species of *Artemisia amygdalina* (Lane 2) (Fig.1). The band corresponding to approximately 200bp fragment is present only in tissue cultured *Artemisia amygdalina* and is missing in other three species of *Artemisia* viz. *Artemisia annua*, wild *Artemisia amygdalina* and *Artemisia absinthium*. However an additional band in the range of 150-200bp is seen only in wild type *Artemisia amygdalina*.

In PCR-ISSR analysis of *Artemisia* species using the primer 5' ACTG ACTG ACTG ACTG ACTG 3' similar kind of band pattern was observed in all the four *Artemisia* species except for the minor variation in the pattern of the tissue cultured species of *Artemisia amygdalina* (Lane 4) (Fig.2). The band corresponding to the 0.3kb fragment which is present in other three species of *Artemisia* viz. *Artemisia annua*, wild *Artemisia amygdalina* and *Artemisia absinthium* is missing in the tissue cultured *Artemisia amygdalina*. However, two prominent bands in the range of 0.15kb and 0.2kb can be seen only in tissue cultured *Artemisia amygdalina*.

DISCUSSION

ISSR markers were used to find out the somaclonal variations among the four samples (wild and tissue cultured *Artemisia amygdalina*, *Artemisia annua* and *Artemisia absinthium*). Two ISSR-primers were used. One primer with sequence (CAC)₃GC was used for the amplification of primer specific sequences. With this primer, sequence similarity was seen in all the four species. Only in tissue cultured species an additional band corresponding to about 0.2kb was seen. This band was absent in all other three species. Also a band corresponding to about 0.5kb-0.2kb was seen in the wild type *Artemisia amygdalina* (Fig 1). The presence of additional 0.2kb band in tissue cultured species shows that an additional sequence corresponding to the primer sequence (CAC)₃GC was present which means that somaclonal variation was present in the tissue cultured species. This variation can prove beneficial in the medicinal property of the plant.

Further to reveal the somaclonal variation at the genetic level a second primer (ACTG)₅ was used for PCR amplification of the four species. Again two prominent bands were seen in the range of 0.5kb and 0.2kb in tissue cultured species. Further a band corresponding to 0.3kb which is present in all three species is found absent in the tissue cultured species (Fig 2). Thus amplification with this primer shows an addition and deletion type of genetic variation in tissue cultured species. This reveals somaclonal variation at the genetic level. Though the regenerated plants were phenotypically normal and essentially identical with their mother plant, they showed genetic variations when subjected to RAPD and ISSR analysis. Similar findings on genomic variation in phenotypically normal regenerants have been well documented in some other plants (Rahman & Rajora 2001; Kawiak & Lojkwaska 2004). Somaclonal variations have been reported in number of species, such as *Populus deltoids* (Rani, *et al.* 1995), *Actinida deliciosa* (Palombi & Damiano 2002).

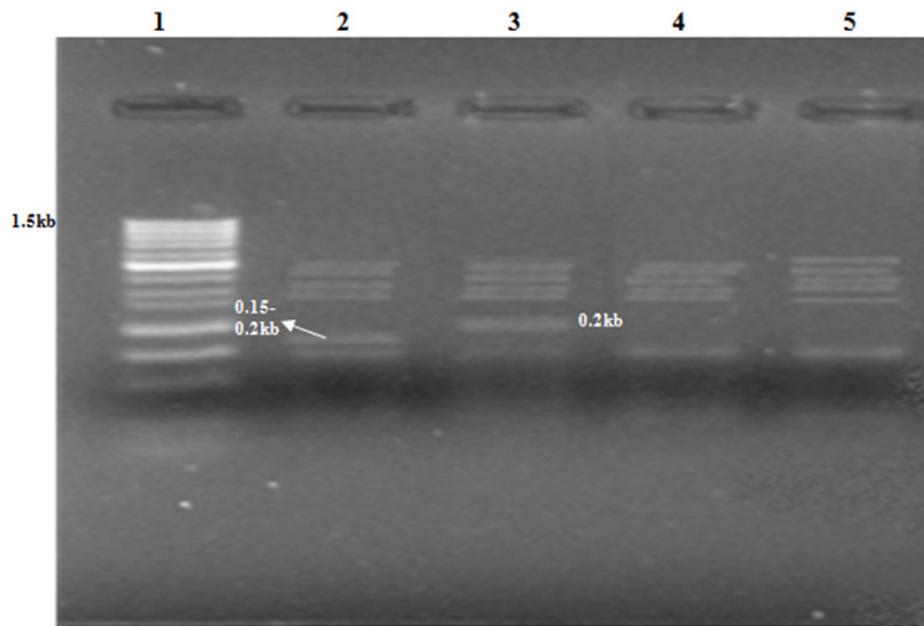


Fig 1: PCR-Inter simple sequence repeats (ISSR) of genomic DNA of *Artemisia* species using primer $(CAC)_3GC$.

- Lane 1 - 50bp DNA ladder
- Lane 2 - Wild *Artemisia amygdalina*
- Lane 3 - Tissue cultured *Artemisia amygdalina*
- Lane 4 - Wild *Artemisia annua*
- Lane 5 - Wild *Artemisia absinthium*

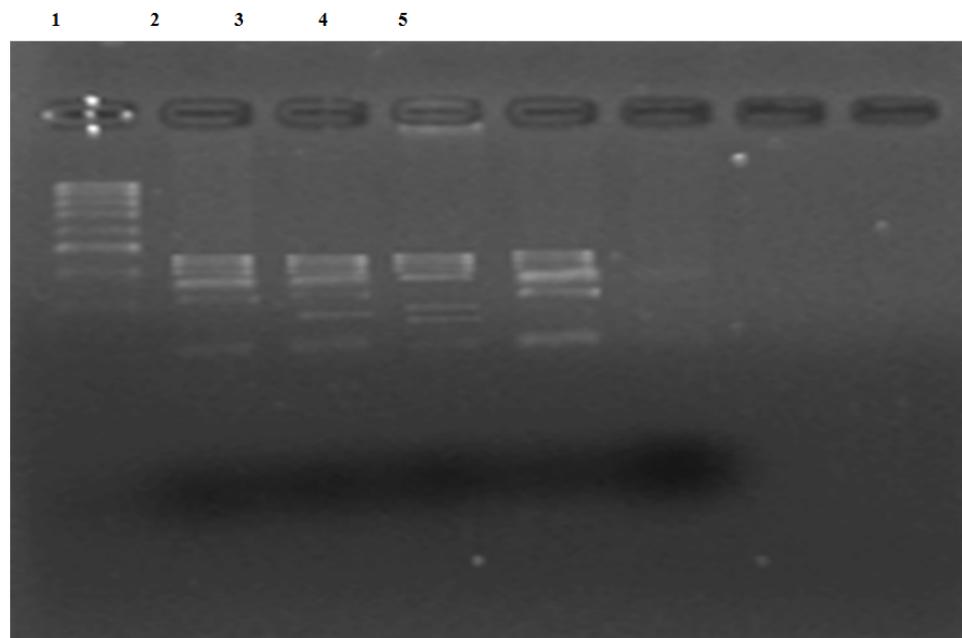


Fig 2: PCR-Inter simple sequence repeats (ISSR) of genomic DNA of *Artemisia* species using primer $(ACTG)_5$.

- Lane 1 - 50bp DNA ladder;
- Lane 2 - Wild *Artemisia annua*
- Lane 3 - Wild *Artemisia amygdalina*
- Lane 4 - Tissue cultured *Artemisia amygdalina*
- Lane 5 - Wild *Artemisia absinthium*

Many reports have indicated the occurrence of somaclonal variation in micropropagated plants of bananas (Krikorian 1989; Schoofs 1992). Roy *et al.* (1992) used RAPD to check the clonal trueness of silver birch (*Betula pedula*). The somaclonal variation occurring in the tissue cultured plant at the genetic level can be proved easily by ISSR technique (Lakshmanan *et al.* 2006).

CONCLUSION

The variations found in the amplification products reveals somaclonal variation in tissue cultured species of *Artemisia amygdalina*. ISSR markers can testify whether the direct regeneration is true to the type or not as in the present study direct regeneration (through nodal explant) has shown somaclonal variation.

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