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In vitro Propagation and Analysis of Genetic Stability of *In vitro* Propagated Plants of Jaspi- A Clonal Rootstock of *Prunus*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors RK and KK designed the study. Authors SM and NS performed the wet lab experiments, statistical analysis, wrote the protocol, wrote the first draft of the manuscript and managed the analyses of the study. Author Shilpa managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

A reproducible protocol for *in vitro* propagation of 'Jaspi'- a *Prunus* rootstock was established. Jaspi is an improved rootstock dwarfing in nature and drought tolerant. The most efficient bud induction medium consisted of Murashige and Skoog (MS) medium fortified with 0.75 mg/l Benzyl Adenine (BA) and 3 mg/l Gibberellic acid (GA_3). After four weeks, the shoot buds were fragmented and transferred to the medium of same composition for *in vitro* shoot multiplication. *In vitro* elongated shoots were successfully rooted and transferred to soil. The genetic stability of micropropagated plants was analysed by RAPD, SSR and ISSR molecular markers. The results indicated that almost no somaclonal variation was detected among the micropropagated plants.

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1. INTRODUCTION

Prunus is a large genus of family Rosaceae which includes plums, cherries, peaches, apricots, and almonds [1]. There are more than 400 species under genus *Prunus* spread throughout the Northern temperate regions of the globe (www.wikipedia.org/wiki/Prunus). Varied rootstocks are used for *Prunus* species on a worldwide basis [2]. Each one has its strengths and limitations for adaptation to different geographic regions. Many clonal rootstocks of *Prunus* had recently been introduced in India, to name a few Myrocal, Jaspi, Julior, Montclar, Ishtara, Cadaman, Citation, etc. All of them had been tried for propagation under field conditions and some of them such as Myrocal, Jaspi and Julior had been found difficult to root and thus, mass propagation is difficult to achieve through conventional methods. Therefore, their *in vitro* multiplication was undertaken. However, genetic fidelity is one of the most important pre-requisites in the micropropagation of any crop species. A major problem encountered with the *in vitro* culture is the presence of somaclonal variations [3] occuring amongst subclones of one parental line.

Though variations can be studied through morphological and biochemical markers also, but DNA markers are stable and not affected by environmental and developmental stages. In case of rootstocks, it becomes difficult to record their morphological traits after grafting. In addition, morphological characters are strongly affected by the environment and also developmental stage of plants [4]. Random Amplified Polymorphic DNA (RAPD), Inter-simple sequence repeats (ISSR) and Simple Sequence Repeats (SSR) are commonly used marker systems since they require only a small amount of DNA sample and are simpler as well as faster and can be used in any laboratory without much of sophisticated infrastructure. At present, RAPD, SSR and ISSR markers have been successfully applied to detect the genetic diversity in micropropagated material in various plants [5-9]. Molecular analyses for diversity have been performed in *Prunus* species using different DNA markers such as RFLPs [10-11], RAPDs [12-13], AFLPs [14-15], RFLPs [16-17], SNPs [15] and SSRs [18-23].

Prunus fruits are cultivated in Himachal Pradesh on seedling rootstocks which are not suitable for high density plantations as they impart excessive vigor. Size controlling rootstocks are the need of hour, in particular, Jaspi an improved clonal rootstock is not only dwarfing in nature, but also drought tolerant, thus, is considered suitable for stone fruits. However, it is not easily propagated by conventional methods of vegetative propagation. Therefore, the present work was undertaken to enhance multiplication of *in vitro* propagation along with assessing genetic stability of its micropropagated plants using RAPD, SSR and ISSR markers.

2. MATERIALS AND METHODS

2.1 *In vitro* Propagation

2.1.1 Source of plant material

Jaspi rootstock being maintained in the fields of Department of Fruit Science, Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (India) formed the source plant material for *in vitro* propagation. The nodal explants were sterilized with 0.3% solution of bavistin (Carbendazim- a fungicide) for 12 minutes in combination with 0.1% solution of $HgCl_2$ for 3 minutes. After sterilization the nodal explants were inoculated on MS medium supplemented with BA at 0.25 mg/l to 2 mg/l, GA_3 at 0.5 mg/l to 3 mg/l and Kn at 0.5 mg/l (Table 1) in combination with sucrose at 20-30 gm/l, agar-agar 8 gm/l and were maintained at a temperature of 25±2 ℃ and 35 µmole m^{-2} s^{-1} photosynthetic photon flux (PPF), white florescent light was emitted by 40 W fluorescent tube lights (Philips, India), programmed for 16/8 hours photoperiod.

The elongated shoots (2.5-3.5 cm in length) were excised from *in vitro* multiplying shoots and cultured in glass tubes (150 mmX10 mm) containing half strength medium with or without 1 mg/l IBA for rooting. The shoots were given a prior dip in 1 ppm IBA for different durations of time. The cultures were maintained under the same culture conditions as above. After rooting the plantlets were transferred to plastic pots containing various autoclaved potting mixtures viz, sand: soil: FYM (Farm Yard Mannure); 1:1:1, sand: soil; 1:1 and coco peat alone and placed under growth conditions of high humidity and light.

2.2 Genetic Stability Analysis

Three different types of markers RAPD, SSR and ISSR were used to study genetic stability of *in vitro* propagated plants. About 2 g of green fresh and healthy leaves were excised both from *in vitro* grown plantlets (samples from plant material being multiplied for the last more than six years) and one sample from field grown parent plant. All the samples were wrapped in aluminium foil. These were labelled properly and stored in deep freezer at -20℃ till further use.

2.3 Isolation of Genomic DNA

Genomic DNA from the collected leaves was isolated by CTAB method of Doyle and Doyle [24], with some modifications wherever required. The quality of the extracted DNA was estimated by agarose gel electrophoresis and quantity was evaluated using picodrop spectrophometer (Picodrop Ltd. Cambridgeshire, UK).

2.4 RAPD Analysis

A total of 16 random 10-mer primers (Metabion International AG, Deutschland, Germany procured through Genaxy, New Delhi, India) were used for RAPD analysis of micropropagated plants and ten of them listed in Table 2. PCR was carried out in 20 µl volume containing 25-35 ng genomic DNA, 1X PCR Buffer A, 2.2 mM $MgCl_2$, 1 mM dNTP, 0.2 µM of primer, 1 U Taq DNA polymerase on a thermal cycler (Multigene, Bangalore, India), programmed for initial denaturation of 3 min at 95℃ followed by 32 cycles each of 30 sec at 94℃, 30 sec at 35℃ and 1 min at 72℃, finally a 10 min extension at 72℃ and lastly was hold at 4℃. The RAPD amplification products were separated by electrophoresis on a 1.2% (w/v) agarose gel in 1X TAE buffer.

2.5 SSR Analysis

SSR analysis of genomic DNA using 10 pairs of SSR primers (Metabion International AG, Deutschland, Germany procured through Genaxy, New Delhi, India) was carried out. PCR protocol was standardized for carrying out the amplification of 23 samples of micropropagated plants and one parent plant. The reaction mixture of 20 µl contained 60 ng genomic DNA, 1X PCR Buffer A, 2.2 mM $MgCl_2$, 1 mM dNTP, 3.0 µM of primer, 1 U Taq DNA polymerase. The PCR cycles were standardized as follows: one initial denaturation cycle of 5 min at 94℃, 40 cycles each of 45 seconds at 95℃, annealing of 45 seconds at primer specific annealing temperature, extension of 45 seconds at 72℃ and a final extension of 10 minute at 72℃. The amplified product was electrophoresed on a 2% (w/v) agarose gel in 1X TAE buffer.

2.6 ISSR Analysis

ISSR pattern for 23 samples of micropropagated plants and one parent plant was studied using 14 ISSR primers (Metabion International AG, Deutschland, Germany procured through Genaxy, India). PCR protocol was standardized for carrying out the amplification. The reaction mixture of 20 µl contained 20 ng genomic DNA, 1X PCR Buffer A, 2.2 mM $MgCl_2$, 1 mM dNTP, 0.2 µM of primer, 1 U Taq DNA polymerase. The PCR cycles for ISSR were standardized as follows: one initial denaturation cycle of 2 minutes at 94℃, 40 cycles each of 10 seconds at 94℃, annealing of 30 seconds at primer

Table 1. *In vitro* bud break of Jaspi on different media combinations based on MS basal salts formulation after two weeks of culturing

Sr.No	Medium code (MS Basal)	Kn mg/l	BA mg/l	GA_3 mg/l	Percentage of buds proliferated
1.	E-1	0.5	2	3	66.70 (54.76)
2.	E-2	0.5	1.5	3	67.33 (55.14)
3.	E-3	0.5	1	3	68.21(55.68)
4.	E-4	-	2	3	74.95(59.97)
5.	E-5	-	1	3	78.93(62.68)
6.	E-6	-	0.75	3	85.10(67.66)
7.	E-7	-	0.50	3	83.22(65.82)
8.	E-8	-	0.25	3	81.55(64.23)
	$CD_{0.05}$		2.45		

Figures in parentheses () are the arc sine transformation of percentage.

Table 2. Primers used to assess genetic stability of *in vitro* propagated plants of Jaspi-*Prunus* rootstock

S.No	RAPD primers used				
	Primer	**Primer sequence 5'→3'**	**Total number of amplified bands**	**Total number of segments**	**Size range of amplified bands in base pairs**
1.	RAPD-A	AATCGGGCT	3	72	500-800
2.	RAPD-C	GGGTAACGC	3	72	200-700
3.	RAPD-D	CAATCGCCGT	4	96	100-500
4.	RAPD-E	TCTGTGCTGG	4	96	500-1000
5.	RAPD-F	TTCCGAACCC	3	72	500-800
6.	RAPD-G	GACCGCTTGT	8	192	100-800
7.	RAPD-H	AGGTGACCGT	7	168	500-1000
8.	RAPD-I	CAAACGTCGG	2	48	2000-3000
9.	RAPD-J	GTTGCGATCC	4	96	700-3000
10.	SIGMA-1	TTTGCTCGGC	1	24	650-700
	Total		38	936	
	SSR primers used				
S.No	**Primer name**	**Primer sequence 5'→3'**	**Total number of amplified bands**	**Total number of segments**	**Size range of amplified bands in base pairs**
1	SSR-1	Forward:GTAACGCTCGCTACCACAAAA Reverse:CCTGCATATCACCACCCAG	1	24	1000-2000
2	SSR-2	Forward:TTCTAATCTGGGCTATGGCG Reverse:GAAGTTCACATTTACGACAGGG	1	24	800-1000
3	SSR-3	Forward:TAAGAGGATCATTTTTGCCTTG Reverse:CCCTGGAGGACTGAGGGT	1	24	700-800
4	SSR-4	Forward:TCCCATAACCAAAAAAAACACC Reverse:TGGAGAAGGGTGGGTACTTG	1	24	1000-2000
5	SSR-5	Forward:TCGGAAACTGGTAGTATGAACAGA Reverse:ATGGGTAGTATGCACAGTCA	2	48	700-1000
6	SSR-6	Forward:ACCACCATTTTGGCTCTCTG Reverse:ACCACCACAACCAAACCATT	2	48	1000-3000
7	SSR-7	Forward:ATAATCCGGCAGGGTCTTA Reverse:TTGGGGTTTGTCAGTATTTTACA	1	24	800-1000
8	SSR-8	Forward:CTGCCGAAAGCATTTTGAAT Reverse:GAGCTCATGGCAACACAGAA	2	48	800-2000

9	SSR-9	Forward:CAACGAGCTCCCATGACTTT Reverse:ACCACCACAACCAAACCATT	1	24	800-1000
10	SSR-10	Forward:GCCAGGAGGCTTTAACCTGT Reverse:TCAGACCCCCTTTCATCATC	1	24	800-1000
	TOTAL		13	312	
		ISSR primers used			
S.No.	**Primer code**	**Primer sequence (5' to 3')**	**Total number of amplified products**	**Total number of amplified segments**	**Size range of amplified bands in base pairs**
1.	ISSR-A	CTCTCTCTCTCTCTCTT	5	120	1000-3000
2.	ISSR-B	CACACACACACACACAT	2	48	800-3000
3.	ISSR-C	TCTCTCTCTCTCTCTCA	2	72	4000-5000
4.	ISSR-D	TCTCTCTCTCTCTCTCG	2	72	700-800
5.	ISSR-E	ACACACACACACACACG	2	96	2000-3000
6.	ISSR-F	GACAGACAGACAGACA	4	120	800-2000
7.	ISSR-G	GTGGTGGTGGTGGTG	3	48	700-1000
8.	ISSR-H	GACGACGACGACGAC	5	120	800-1000
9.	ISSR-I	CACACACACACACACACG	6	144	800-3000
10.	ISSR-J	GAGAGAGAGAGAGAGACG	2	48	600-1000
11.	ISSR-L	GACAGACAGACAGACA	1	24	800-1000
12.	ISSR-M	ACACACACACACACAC	1	24	3000-4000
13.	ISSR-O	CACACACACACACACAGC	1	24	650-700
14.	ISSR-P	GAGAGAGAGAGAGATA	3	72	1000-3000
	TOTAL		39	1032	

specific annealing temperature, extension of 65 seconds at 72℃ and a final extension of 10 minute at 72℃ and last was hold at 4℃. Amplification products were separated by electrophoresis on a 1.2% (w/v) agarose gel in 1X TAE buffer.

In all the marker systems used in the study, the amplification for PCR was carried out in thermal cycler (Multigene, Bangalore, India). The size of the amplified product was determined by co-electrophoresis of standard molecular weight marker (double digest of HindIII/EcoRI Bangalore Genei, India). DNA profiles were visualized on a UV-transilluminator and photographed using gel documentation system (Syngene, Cambridge, UK).

3. RESULTS

Initial proliferation of buds was found to be best on MS medium supplemented with 0.75 mg/l BA and 3 mg/l GA_3, giving 85.10% of bud break after two weeks of culturing (Table 1).

The proliferation rate increased with the increase in culturing time. In about four weeks, the highest percentage reached upto 95%. The sprouted buds were transferred to fresh medium of same composition and the medium, which proved best for bud sprouting, found to be the best for further shoot multiplication upon successive subculturing. At the end of first culture of four weeks, the maximum average number of *in vitro* shoots obtained per explant was recorded to be 7.14. This number kept on increasing and as much as average number of 25 shoots were obtained (data not shown). We have recorded that after sixth or seventh subculture, the leaves of *in vitro* multiplying shoots turn yellowish and rate of multiplication slows down. However, it again picks up after eighth to tenth subculture and the process goes on. After about six years of start of initial culturing we are carrying on *in vitro* multiplication without any apparent variation though, we started culturing afresh also to enhance the overall multiplication rate.

The temperature played an important role on promoting shoot multiplication. It has been observed that rate of shoot multiplication is very sensitive to increase or decrease in temperature even by two degrees. The optimum temperature has been recorded to be 24℃ for over all *in vitro* work in rootstock "Jaspi". *In vitro* root induction has been recorded to be best on half strength MS basal + 1 mg/l IBA after a prior quick dip of shoots in 1 ppm IBA. Percent survival in different potting mixtures is also a temperature sensitive process. In the month of November 100% survival of micropropagated plantlets was observed in cocopeat. Hardened plants were then transferred to field soil with more than 90% survival. The survival rate of micropropagated plants on autoclaved sand: soil: FYM; 1:1:1 and sand: soil; 1:1 was recorded to be about 60%.

In the second objective we aimed at assessing genetic stability of long term micropropagated plants of *Prunus* rootstock – Jaspi, using three different DNA markers systems- namely RAPD, SSR and ISSR.

Our research group followed CTAB method for DNA isolation with some modifications whenever needed. The presence of high molecular weight band on agarose gel indicated good quality of DNA.

After assessing quality of DNA the quantity was assessed on picodrop-spectrophotometer. After that it was standardized to use 20 ng/µl of DNA for RAPD analysis, whereas 50-70 ng DNA was used for both SSR as well as ISSR studies. The PCR protocol for amplification of genomic DNA was standardized by varying the concentration of different components.

RAPD, ISSR and SSR analysis of 23 samples taken randomly from *in vitro* raised plantlets of *Prunus* rootstock and a parent plant, was carried out. A total of eleven RAPD primers, 10 SSR primers and 14 ISSR primers were used for DNA amplification. Out of 11 RAPD primers, as many as ten primers had shown scorable banding patterns. The 10 RAPD primers yielded 38 scorable bands with an average of 3.80 bands per primer. No genetic variation was detected in the micropropagated plants.

All the ten SSR primers had shown scorable banding patterns in all the 23 plants selected randomly. The 10 SSR primers generated 13 scorable bands with an average of 1.3 bands per primer. Similar to RAPD analysis all SSR loci detected no genetic variation among the clones.

All the 14 ISSR primers produced 39 scorable bands and an average of 2.78 bands per primer. All the 14 primers produced monomorphism as in case of RAPD and SSRs. The monomorphism given by all the primers indicated a high degree of genetic fidelity amongst the *in vitro* raised plants.

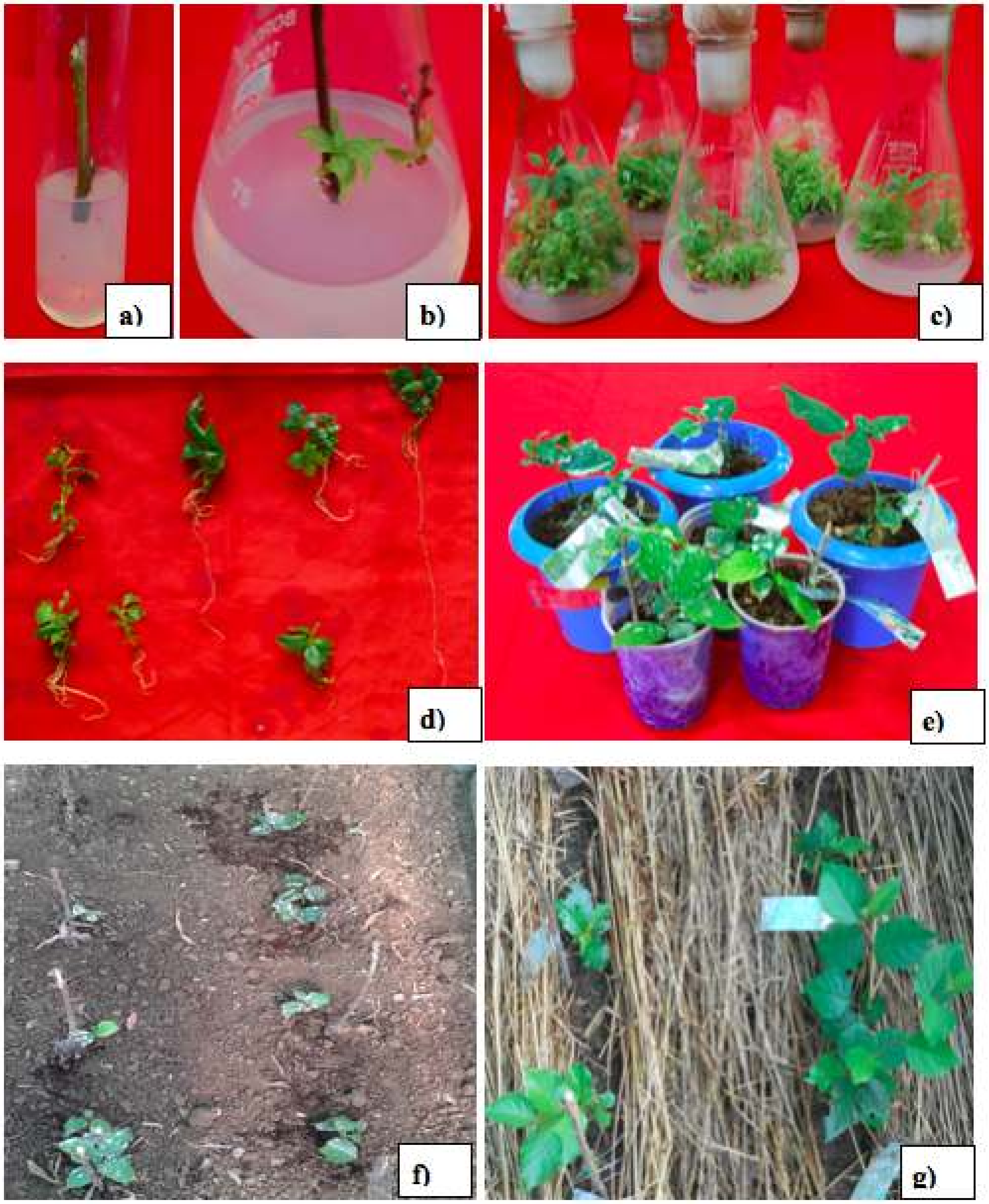


Fig. 1. *In vitro* propagation of "Jaspi" a) initiation of bud break b)sprouted buds c) multiplication of Jaspi shoots d) *in vitro* rooted plantlets e) *in vitro* propagated plants being hardened in pots f) hardened plants transferred to soil g) hardened plants growing in the field after two months of transfer

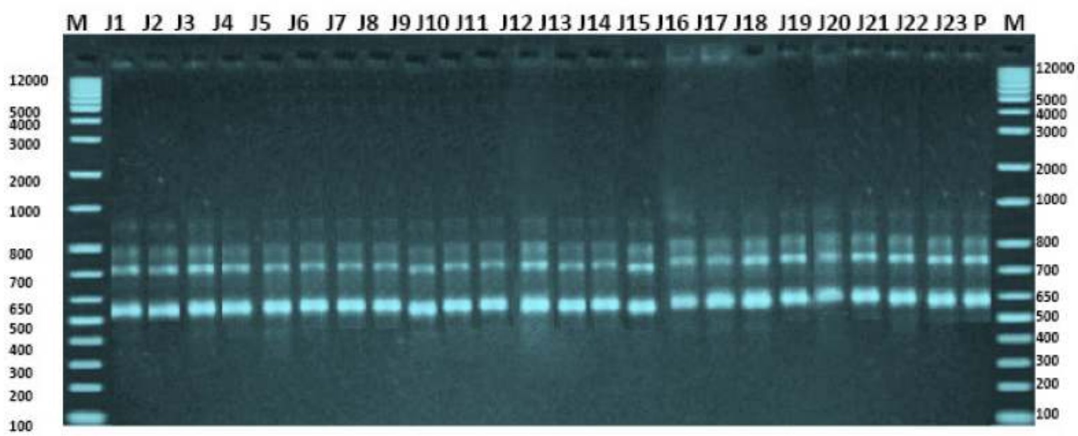
4. DISCUSSION

The assessment of genetic stability of micropropagated plant material is essential for maintenance of trueness-to-type. This can be achieved by developing the micropropagation protocols based upon axillary branching. But there are some reports which document the occurrence of somaclonal variation [25-28] among plants derived through enhanced axillary branching cultures. Therefore, irrespective of the method of micropropagation, there should be quality check for genetic uniformity of micropropagated plants.

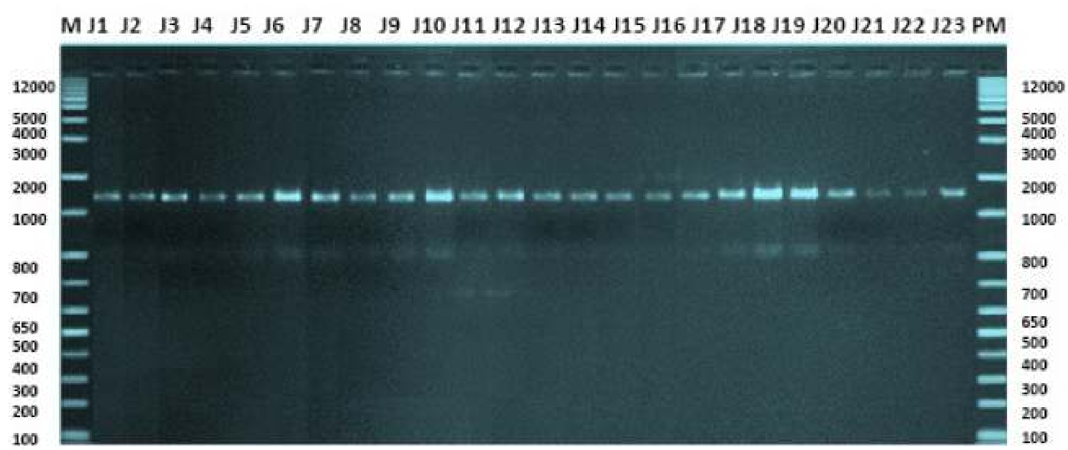
There are reports where *Prunus* plants were established and multiplied on MS-basal medium supplemented with different growth regulators like GA_3, BA, IBA, TDZ [29-31]. In present study, protocol for establishment of *in vitro* cultures has been developed. Bud sticks, each having 3-4 buds, were taken from plants growing in the fields and highest sprouting rate (85.10%) of the explants was found to be in MS medium supplemented with 0.75 mg/l BA and 3 mg/l GA_3 after two weeks of culturing. Many workers [32-33] reported the use of BA and Kinetin for shoot proliferation from explants of various rootstocks of *Prunus* species. Reeves *et al* [34] found that addition of GA_3 to the medium, caused elongation of shoots of peach and plum rootstocks.

4.1 Assessment of Trueness-to-Type

To compare the efficiency of the use of single versus multiple markers, we assessed genetic variation using RAPD, SSR and ISSR markers and evaluated how well these markers confirmed genetic stability of micropropagated plants of Jaspi rootstock. Using only one type of marker system to assess genetic variation sometimes gives results that have been questioned in terms of efficiency and reliability as compared to the combined use of different markers.



Primer- RAPD-E
5' CAATCGCCGT3'
No. of scorable bands-4



Primer – SSR-4
F:5' TCCCATAACCAAAAAAAACACC3'
R:5' TGGAGAAGGGTGGGTACTTG3'
No. of scorable bands-1

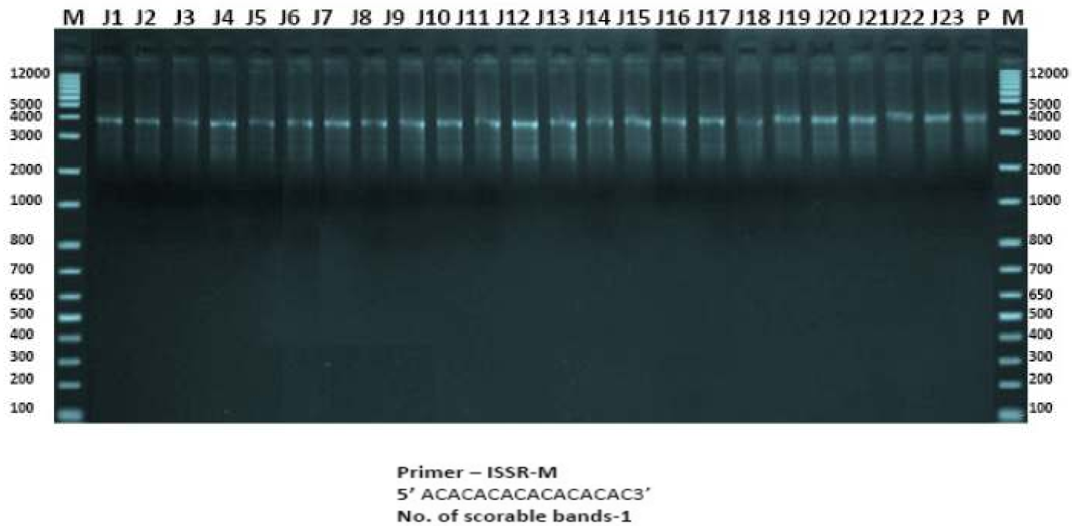


Fig. 2. RAPD, SSR and ISSR pattern
J1-J23: 23 randomly selected micropropagated plants; P: Parent plant
M: Known Molecular weight ladder 100 bp

A total of 35 primers of three different types of marker systems viz. RAPD, SSR and ISSR were used to assess genetic stability of micropropagated plants. The concentration of various PCR components was standardized separately for all kind of primers used and thermal profiles were also different for the three primer types, since it depends on annealing temperature, which is essentially different for different primer types. All the bands generated using RAPD primers were found to be monomorphic. The monomorphic banding pattern of *in vitro* rootstock and field grown parent plant shows that tissue culture raised rootstock is genetically identical to the rootstock grown in field conditions and hence are genetically stable even after a period of six years of culturing. Hashmi et al. [25] demonstrated the feasibility of using RAPD markers to identify somaclonal variants of peach and provided an evidence for the existence of genetic differences among the variants. However, Rout and Das [35] concluded from the genetic fidelity studies of micropropagated plants of *Plumbago zeylanica* that micropropagated plants were monomorphic and similar to field grown parent plant using 20 RAPD primers. Similarly utility of RAPD as a means of molecular analysis of *in vitro* regenerated plants has been very well documented by many workers [26,36-41]. In addition to RAPDs, two other marker systems i.e. SSR and ISSR have been used in present investigation to study the genetic stability of long term micropropagated plants of the *Prunus* rootstock. The SSR and ISSR banding pattern of all 23 *in vitro* samples were compared with parent plant. The 13 bands and 39 bands given by SSR and ISSR respectively were recorded to be monomorphic and found to be at the same level as those of parent plant. SSR analysis for micropropagated sugarcane plants were done by Srivastava et al [42] to assess the genetic fidelity. The amplified products exhibited monomorphism among all the *in vitro* raised plants and had been found to be similar to those of parent plant. Similar work for assessment of stability has been done [43] on olive species and studies suggested genetic uniformity throughout the process. Li et al [44] demonstrated the use of ISSR primers to study the genomic fidelity of micropropagated plants of *Robinia psedoacacia* and found monomorphism. Likewise, various studies carried out by different workers to assess the trueness-to-type of micropropagated plants using ISSR primers and almost all were found to give maximum percentage of monomorphism [45] on *Prunus mume*; [46] on *Dictyospermum ovalifolium*, [47] on *Fragaria ananassa Duch.* and [48] on *Nothapodytes foetida*.

5. CONCLUSION

However, the *in vitro* propagation protocol for *Prunus* rootstock has been demonstrated to be reliable, reproducible and efficient over a period of more than six years. RAPD, SSR and ISSR techniques have been applied to investigate genetic stability and are found to be efficient and reliable. The present results indicated no variation amongst the *in vitro* propagated plants and the results have been found to be satisfactory for displaying trueness-to-type of micropropgated plant material with parent plant. Hence in conclusion all three kinds of markers i.e RAPD, SSR and ISSR can be successfully applied to determine the genetic integrity of micropropagated plants of Jaspi rootstock and the protocol developed for micropropagation can be used over a long period without the risk of somaclonal variations.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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