## 1 In vitro propagation and analysis of genetic stability of in vitro propagated plants of Jaspi- a clonal rootstock

2 of Prunus

# 3 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<sub>3</sub>). 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.

11 Keywords: genetic stability; Jaspi; clonal rootstock; Prunus

# 12 INTRODUCTION

13 Prunus is a large genus of family Rosaceae which includes plums, cherries, peaches, apricots, and almonds 14 (Rehder 1940). There are more than 400 species under genus Prunus spread throughout the Northern temperate 15 regions of the globe (www.wikipedia.org/wiki/Prunus). Varied rootstocks are used for Prunus species on a 16 worldwide basis (Rom 1982). Each one has its strengths and limitations for adaptation to different geographic 17 regions. Many clonal rootstocks of *Prunus* had recently been introduced in India, to name a few Myrocal, Jaspi, 18 Julior, Montclar, Ishtara, Cadaman, Citation, etc. All of them had been tried for propagation under field conditions 19 and some of them such as Myrocal, Jaspi and Julior had been found difficult to root and thus, mass propagation is 20 difficult to achieve through conventional methods. Therefore, their *in vitro* multiplication was undertaken. However, 21 genetic fidelity is one of the most important pre-requisites in the micropropagation of any crop species. A major 22 problem encountered with the *in vitro* culture is the presence of somaclonal variations (Larkin and Scowcroft 1981) 23 occuring amongst subclones of one parental line.

24 Though variations can be studied through morphological and biochemical markers also, but DNA markers 25 are stable and not affected by environmental and developmental stages. In case of rootstocks, it becomes difficult to 26 record their morphological traits after grafting. In addition, morphological characters are strongly affected by the 27 environment and also developmental stage of plants (Casas et al 1999). Random Amplified Polymorphic DNA 28 (RAPD), Inter-simple sequence repeats (ISSR) and Simple Sequence Repeats (SSR) are commonly used marker 29 systems since they require only a small amount of DNA sample and are simpler as well as faster and can be used in 30 any laboratory without much of sophisticated infrastructure. At present, RAPD, SSR and ISSR markers have been 31 successfully applied to detect the genetic diversity in micropropagated material in various plants [Carvalho et al 32 2004; Martins et al 2004; Ramage et al 2004; Modgil et al 2005; Kaur et al 2009]. Molecular analyses for diversity 33 have been performed in Prunus species using different DNA markers such as RFLPs (Kaneko et al 1986; Uematsu 34 et al 1991), RAPDs (Gogorcena and Parfitt 1994; Lu et al 1996; Casas et al 1999), AFLPs (Aradhya et al 2004; Fang 35 et al 2006), RFLPs (Badenes and Parfitt 1995; Bouhadida et al 2007), SNPs (Fang et al 2006) and SSRs (Testolin et 36 al 2004; Mnejja et al 2005; Messina et al 2004; Clarke and Tobutt 2003; Aranzana et al 2002; Mnejja et al 2004).

37 Prunus fruits are cultivated in Himachal Pradesh on seedling rootstocks which are not suitable for high 38 density plantations as they impart excessive vigor. Size controlling rootstocks are the need of hour, in particular, 39 Jaspi an improved clonal rootstock is not only dwarfing in nature, but also drought tolerant, thus, is considered 40 suitable for stone fruits. However, it is not easily propagated by conventional methods of vegetative propagation. 41 Therefore, the present work was undertaken to enhance multiplication of *in vitro* propagation along with assessing

42 genetic stability of its micropropagated plants using RAPD, SSR and ISSR markers.

### 43 MATERIAL AND METHODS

### 44 IN VITRO PROPAGATION

## 45 Source of plant material

46 Jaspi rootstock being maintained in the fields of Department of Fruit Science, Dr Y S Parmar University of 47 Horticulture and Forestry, Nauni, Solan (India) formed the source plant material for in vitro propagation. The nodal 48 explants were sterilized with 0.3% solution of bavistin (Carbendazim- a fungicide) for 12 minutes in combination 49 with 0.1% solution of HgCl<sub>2</sub> for 3 minutes. After sterilization the nodal explants were inoculated on MS medium 50 supplemented with BA at 0.25 mg/l to 2 mg/l, GA<sub>3</sub> at 0.5 mg/l to 3 mg/l and Kn at 0.5 mg/l (Table 1) in combination 51 with sucrose at 20-30 gm/l, agar-agar 8 gm/l and were maintained at a temperature of 25±2 °C and 35 µmole m<sup>-2</sup> s<sup>-1</sup> 52 photosynthetic photon flux (PPF), white florescent light was emitted by 40 W fluorescent tube lights (Philips, India), 53 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 (150mmX10mm) 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.

# 60 Genetic stability analysis

Three different types of markers RAPD, SSR and ISSR were used to study genetic stability of *in vitro* propagated plants. About 2g 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°C till further use.

### 66 Isolation of genomic DNA

67 Genomic DNA from the collected leaves was isolated by CTAB method of Doyle and Doyle (1987), with
68 some modifications wherever required. The quality of the extracted DNA was estimated by agarose gel
69 electrophoresis and quantity was evaluated using picodrop spectrophometer (Picodrop Ltd. Cambridgeshire, UK).

# 70 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
PCR was carried out in 20µl volume containing 25-35 ng genomic DNA, 1X PCR Buffer A, 2.2 mM MgCl<sub>2</sub>, 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°C followed by 32 cycles each of 30 sec at 94°C, 30 sec at 35°C
and 1 min at 72°C, finally a 10 min extension at 72°C and lastly was hold at 4°C. The RAPD amplification products
were separated by electrophoresis on a 1.2% (w/v) agarose gel in 1X TAE buffer.

## 78 SSR analysis

79 SSR analysis of genomic DNA using 10 pairs of SSR primers (Metabion International AG, Deutschland, 80 Germany procured through Genaxy, New Delhi, India) was carried out. PCR protocol was standardized for carrying 81 out the amplification of 23 samples of micropropagated plants and one parent plant. The reaction mixture of 20 µl 82 contained 60 ng genomic DNA, 1X PCR Buffer A, 2.2 mM MgCl<sub>2</sub>, 1 mM dNTP, 3.0 µM of primer, 1 U Taq DNA 83 polymerase. The PCR cycles were standardized as follows: one initial denaturation cycle of 5 min at 94°C, 40 cycles 84 each of 45 seconds at 95°C, annealing of 45 seconds at primer specific annealing temperature, extension of 45 85 seconds at 72°C and a final extension of 10 minute at 72°C. The amplified product was electrophoresed on a 2% 86 (w/v) agarose gel in 1X TAE buffer.

## 87 ISSR analysis

88 ISSR pattern for 23 samples of micropropagated plants and one parent plant was studied using 14 ISSR 89 primers (Metabion International AG, Deutschland, Germany procured through Genaxy, India). PCR protocol was 90 standardized for carrying out the amplification. The reaction mixture of 20 µl contained 20 ng genomic DNA, 1X 91 PCR Buffer A, 2.2 mM MgCl<sub>2</sub>, 1 mM dNTP, 0.2 µM of primer, 1 U Taq DNA polymerase. The PCR cycles for 92 ISSR were standardized as follows: one initial denaturation cycle of 2 minutes at 94°C, 40 cycles each of 10 seconds 93 at 94°C, annealing of 30 seconds at primer specific annealing temperature, extension of 65 seconds at 72°C and a 94 final extension of 10 minute at 72°C and last was hold at 4°C. Amplification products were separated by 95 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).

# 100 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<sub>3</sub>, giving 85.10% of bud break after two weeks of culturing (Table 1).

103 The proliferation rate increased with the increase in culturing time. In about four weeks, the highest 104 percentage reached upto 95%. The sprouted buds were transferred to fresh medium of same composition and the 105 medium, which proved best for bud sprouting, found to be the best for further shoot multiplication upon successive 106 subculturing. At the end of first culture of four weeks, the maximum average number of *in vitro* shoots obtained per 107 explant was recorded to be 7.14. This number kept on increasing and as much as average number of 25 shoots were 108 obtained (data not shown). We have recorded that after sixth or seventh subculture, the leaves of *in vitro* multiplying 109 shoots turn yellowish and rate of multiplication slows down. However, it again picks up after eighth to tenth 110 subculture and the process goes on. After about six years of start of initial culturing we are carrying on in vitro 111 multiplication without any apparent variation though, we started culturing afresh also to enhance the overall 112 multiplication rate.

113 The temperature played an important role on promoting shoot multiplication. It has been observed that rate 114 of shoot multiplication is very sensitive to increase or decrease in temperature even by two degrees. The optimum 115 temperature has been recorded to be 24°C for over all in vitro work in rootstock "Jaspi". In vitro root induction has 116 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. 117 Percent survival in different potting mixtures is also a temperature sensitive process. In the month of November 118 100% survival of micropropagated plantlets was observed in cocopeat. Hardened plants were then transferred to 119 field soil with more than 90% survival. The survival rate of micropropagated plants on autoclaved sand: soil: FYM; 120 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.

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## 141 **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 (Hashmi et al 1997; Zucchi et al 2002; Guo et al 2006 and Ngezahayo et al 2006) 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.

148 There are reports where *Prunus* plants were established and multiplied on MS-basal medium supplemented 149 with different growth regulators like GA<sub>3</sub>, BA, IBA, TDZ (Zotto and Docampo, 1997; Pruski et al 2000 and Ruzic et 150 al 2010). In present study, protocol for establishment of *in vitro* cultures has been developed. Bud sticks, each 151 having 3-4 buds, were taken from plants growing in the fields and highest sprouting rate (85.10%) of the explants 152 was found to be in MS medium supplemented with 0.75 mg/l BA and 3 mg/l GA<sub>3</sub> after two weeks of culturing. 153 Many workers (Bondok et al 1989; Ambrozic et al 1992) reported the use of BA and Kinetin for shoot proliferation 154 from explants of various rootstocks of *Prunus* species. Reeves et al., 1985 found that addition of GA<sub>3</sub> to the 155 medium, caused elongation of shoots of peach and plum rootstocks.

# 156 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.

162 A total of 35 primers of three different types of marker systems viz. RAPD, SSR and ISSR were used to 163 assess genetic stability of micropropagated plants. The concentration of various PCR components was standardized 164 separately for all kind of primers used and thermal profiles were also different for the three primer types, since it 165 depends on annealing temperature, which is essentially different for different primer types. All the bands generated 166 using RAPD primers were found to be monomorphic. The monomorphic banding pattern of *in vitro* rootstock and 167 field grown parent plant shows that tissue culture raised rootstock is genetically identical to the rootstock grown in 168 field conditions and hence are genetically stable even after a period of six years of culturing. Hashmi et al 1997 169 demonstrated the feasibility of using RAPD markers to identify somaclonal variants of peach and provided an

170 evidence for the existence of genetic differences among the variants. However, Rout and Das, 2002 concluded from 171 the genetic fidelity studies of micropropagated plants of *Plumbago zeylanica* that micropropagated plants were 172 monomorphic and similar to field grown parent plant using 20 RAPD primers. Similarly utility of RAPD as a means 173 of molecular analysis of *in vitro* regenerated plants has been very well documented by many workers (Zucchi et al 174 2002; Kawaiak and Ojakaska 2004; Luisa et al 2004; Nas et al 2006; Wen and Deng 2005; Lattoo et al 2006 and 175 Reddampalli et al 2007; Kaur et al 2009). In addition to RAPDs, two other marker systems i.e. SSR and ISSR have 176 been used in present investigation to study the genetic stability of long term micropropagated plants of the Prunus 177 rootstock. The SSR and ISSR banding pattern of all 23 in vitro samples were compared with parent plant. The 13 178 bands and 39 bands given by SSR and ISSR respectively were recorded to be monomorphic and found to be at the 179 same level as those of parent plant. SSR analysis for micropropagated sugarcane plants were done by Srivastava et 180 al 2005 to assess the genetic fidelity. The amplified products exhibited monomorphism among all the *in vitro* raised 181 plants and had been found to be similar to those of parent plant. Similar work for assessment of stability has been 182 done by Gina et al 2010 on olive species and studies suggested genetic uniformity throughout the process. Li et al 183 2006 demonstrated the use of ISSR primers to study the genomic fidelity of micropropagated plants of Robinia 184 psedoacacia and found monomorphism. Likewise, various studies carried out by different workers to assess the 185 trueness- to-type of micropropagated plants using ISSR primers and almost all were found to give maximum 186 percentage of monomorphism (Guo Gui et al 2007 on Prunus mume; Chandrika et al 2008 on Dictyospermum 187 ovalifolium, Debnath 2009 on Fragaria ananassa Duch. and Chandrika et al 2010 on Nothapodytes foetida.

# 188 CONCLUSION

189 However, the *in vitro* propagation protocol for *Prunus* rootstock has been demonstrated to be reliable, 190 reproducible and efficient over a period of more than six years. RAPD, SSR and ISSR techniques have been applied 191 to investigate genetic stability and are found to be efficient and reliable. The present results indicated no variation 192 amongst the *in vitro* propagated plants and the results have been found to be satisfactory for displaying trueness-to-193 type of micropropgated plant material with parent plant. Hence in conclusion all three kinds of markers i.e RAPD, 194 SSR and ISSR can be successfully applied to determine the genetic integrity of micropropagated plants of Jaspi 195 rootstock and the protocol developed for micropropagation can be used over a long period without the risk of 196 somaclonal variations.

## **197 REFERENCES**

- Ambrozic, T.B. Smole, J. and Siftar, A. 1992. Micropropagation of a plum ecotype (*Prunus domestica L.*) as
   rootstock for apricots. *Acta-Hort*. 300: 111-114.
- Aradhya, M. Weeks, C. and Simon, C.J. 2004. Molecular characterization of variability and relationships among
   seven cultivated and selected wild species of *Prunus* L. using amplified fragment length polymorphism. *Sci. Hort.* 103: 131-144.
- Aranzana, M.J. Garcia, M.J. Carbo, J. and Aru, S.P. 2002. Development and variability of microsatellite markers in
   peach. *Plant Breed.* 121: 87-92.

- Badenes, M.L. and Parfitt, D.E. 1995. Phlogenetic relationships of cultivated *Prunus* species from an analysis of
   chloroplast DNA variation. *Theor App Genet*. 90: 1035-1041.
- Bondok, A. Z. Agamy, S. Z. and Gomaa, A. H. 1989. *In vitro* propagation of Mariana 2624 plum rootstock. *Egypt J Hort.* 16(1): 9-16.
- Bouhadida, M. Martin, J.P. Eremin, G. Pinochet, J. Moreno, M.A. and Gogorcena, Y. 2007. Chloroplast DNA
   diversity in *Prunus* and its implication on phylogenetic relationships. *J Am Soc Hort Sci.* 132: 670-679.
- Carvalho. Luisa, C. Goulao, L. Oliveira, C. Goncalves, J.C. and Amanico, S. 2004. RAPD assessment for
   identification of clonal identity and genetic stability of *in vitro* propagated chestnut hybrids. *Plant Cell, Tiss* Org Cult. 77(1): 23-27.
- Casas, A.M. Igartua, E. Balaguer, G. and Moreno, M.A. 1999. Genetic diversity of *Prunus* rootstocks analysed by
   RAPD markers. *Euphytica*. 110: 139-149.
- Chandrika, M. Rai, R.V. Thoyajaksha, A. and Ramachandra, K.K. 2008. Assessment of genetic stability of *in vitro* grown *Dictyospermum ovalifolium. Biol Plant.* 52: 735–739.
- Chandrika, M. Rai, R.V. and Thoyajaksha, A. 2010. ISSR marker based analysis of micropropagated plantlets of *Nothapodytes foetida. Biol Plant.* 54(3): 561-565.
- Clarke, J.B. and Tobutt, K.R. 2003. Development and characterization of polymorphic microsatellites from *Prunus avium* 'Napoleon'. *Mol Ecol Notes*. 3: 578-580.
- Debnath, S.C. Khanizadeh, S. Jamieson, A.R. and Kempler, C. 2009. Inter Simple Sequence Repeat (ISSR) markers
   to assess genetic diversity and relatedness within strawberry genotypes. *Can J Plant Sci.* 88: 313-322.
- Doyle, J.J. and Doyle, J.L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue.
   *Phytochem Bull.* 19: 11-15.
- Fang, J. Twito, T. Zhang, Z. and Chao, C.C.T. 2006. Genetic relationships amongfruting-mei (*Prunus mume* Sieb.
   Et Zucc.) cultivars evaluated with AFLP and SNP markers. *Genome*. 49: 1256-1264.
- Gina, B. Lopes, T. Loureins, J. Rodriguez, E. and Santos, C. 2010. Assessment of genetic stability of two
   micropropagated wild olive species using flow cytometry and microsatellite markers. *Trees.* 24(4): 723-732.
- Gogorcena, Y. and Parfitt, D.E. 1994. Evaluation of RAPD marker consistency for detection of polymorphism in apricot. *Sci Horti*. 59: 163-167.
- Guo, W.L. Gong, L. Ding, Z.F. Li, Y.D. Li, F.X. Zhao, S.P. and Liu, B. 2006. Genomic instability in phenotypically
   normal regenerants of medicinal plant *Codonopsis lanceolata Benth. Et Hook. f.*, as revealed by ISSR and
   RAPD markers. *Plant Cell Rep.* 25: 896-906.
- GuoGui, N. Fan, X.L. Huang, W.J. Bao, M.Z. and Zhang, J.B. 2007. Micropropagation of six Prunus mume
   cultivars through axillary shoot proliferation, and ISSR analysis of cloned plants. *Acta Biol Crac Ser Bot.* 49(1):
   25-31.
- Hashmi, G. Huetell, R. Meyer, R. Krusberg, L. and Hammerschlag, F. 1997. RAPD analysis of somaclonal
   variations derived from embryo callus cultures of peach. *Plant Cell Rep.* 16: 624-627.
- Kaneko, T. Terachi, T. and Tsunewaki, K. 1986. Studies on the origin of crop species by restriction endonuclease
   analysis of organellar DNA. II. Restriction analysis of ctDNA of 11 *Prunus* species. *Jpn J Genet.* 61: 157-168.

- Kaur, R. Panwar, N. Saxena, B. Raina, R. and Bhardwaj. S.V. 2009. Genetic stability in long term micropropagated
   plants of *Gentiana kurroo*. An endangered medicinal plant. *J New Seeds* 10(4): 236-244.
- Kawaiak, A. and Ojakaska, E. 2004. Application of RAPD in determination of genetic fidelity in micropropagated
   *Drosera* plantlets. *In vitro Cell Dev Biol Plant*. 40(6): 592-595.
- Larkin, P.J. and Scowcroft, W.R. 1981. Somaclonal variation- a novel source of variability from cell cultures for
   plant improvement. *Theor App Genet.* 60: 197-214.
- Lattoo, S.K. Bamotra, S. Dhar, R.S. Khan, S. and Dhar A.K. 2006. Rapid plant regeneration and analysis of genetic
   fidelity of *in vitro* derived plants of *Chlorophytum arundinaceum Baker-* an endangered medicinal herb. *Plant Cell Rep.* 25(6): 499-506.
- Li, Y. Guo, W. Gong, L. Li, T. Dong, Y. and Liu, B. 2006. Efficient micropropagation of *Robinia ambigua var*.
   *Idahoensis Idaho Locust* and detection of genomic variation by ISSR markers. *Plant Cell, Tiss Org Cult.* 84(3): 343-351.
- Lu, Z.X. Reighard, G.L. Baird, W.V. Abott, A.G. and Rajapakse, S. 1996. Identification of peach rootstock cultivars
   by RAPD markers. *HortSci.* 31: 127-129.
- Luisa, C.C. Luiis, G. Cristina, O. Josa, C.G. and Sara, A. 2004. RAPD assessment for identification of clonal
   identity and genetic stability of *in vitro* propagated chestnut hybrids. *Plant Cell, Tiss Org Cult* 77(1): 23-27.
- Martins, M. Sarmento ,D. and Oliveira, M.M. 2004. Genetic stability of micropropagated almond plantlets as
   assessed by RAPD and ISSR markers. *Plant Cell Rep* 23(7): 492-496.
- Messina, R. Lain, O. Marrazzo, M.T. Ciprani, G. and Testolin, R. 2004. New set of microsatellite loci isolated in apricot. *Mol Ecol Notes* 5: 531-535.
- Mnejja, M. Gracia-Mas, M. Howad, W. and Arus, P. 2005. Development and transport ability across *Prunus* species
   of 42 polymorphic almond microsatellites. *Mol Ecol Notes* 5: 531-535.
- Mnejja, M. Gracia-Mas, M. Howad, W. Badenes, M.L. and Arus, P. 2004. Simple sequence repeat (SSR) markers of
   Japanese plum (*Prunus salicina*) are highly polymorphic and transferable to peach and almond. *Mol Ecol Notes* 4: 163-166.
- Modgil, M. Mahajan, K. Chakarbarti, S.K., Sharma, D.R. and Sobti, R.C. 2005. Molecular analysis of genetic
   stability in micropropagated apple rootstock MM106. *Sci Hort*. 104(2): 151-160.
- 269 Nas, M.N. Mutlu, N. and Read, P.E. 2004. RAPD DNA analysis of long term cultured hybrid hazelnut. *Hort Sci* 39(5): 1079-1082.
- Ngezahayo, F. Guo, W.L. Gong, L. Li, F.X. Liu, B. and Dong, Y. 2006. Genomic variation in micropropagated
   Robinia ambigua 'idahoensis' revealed by RAPD markers. *Hort Sci.* 41(6):1466-1468.
- Pierik, R.L.M. 1991. Commercial aspects of micropropagation. In: Prakash J and Pierik R L M. eds. Horticulture
   new technologies and applications.Kluwer Academic, Dordrecht. pp 141-153
- Pruski, K..W. Lewis, T. Astatkie, T. and Nowak, J. 2000. Micropropagation of Chokecherry and Pincherry cultivars.
   *Plant Cell Tiss Org Cult.* 63: 93-100.
- 277

- 278 Ramage, C.M. Borda, A.M. Hamill, S.D. and Smith, M.K. 2004. A simplified PCR test for early detection of dwarf
  279 off-types in micopropagated Cavendish banana (*Musa spp. AAA*). *Sci Hort.* 103(1): 145-151.
- Reddampalli, V.S. Lakshmanan, V. and Neelwarne, B. 2007. Genetic fidelity of long term micropropagated shoot
   cultures of vanilla (*Vanilla plantifolia Andrews*) as assessed by molecular markers. *Biotechnol J* 2(8): 1007 1013.
- Reeves, D. W. Couvillon, G. A. and Horton, B. D. 1985. Effect of gibberellic acid (GA<sub>3</sub>) on elongation and rooting
   of 'St. Julien A' rootstock *in vitro*. *Sci Hort*. 26(3): 253-259.
- 285 Rehder, A. 1940. Manual of cultivated trees and shrubs. 2nd ed. Macmillan Co., New York, 212 p.
- 286 Rom, R.C. 1982. A new philosophy for peach rootstocks development. Fruit Variety Journal 36: 34-37.
- 287 Rom, R.C. 1984. A new generation of peach rootstocks. *In*: Proceedings of the 43rd National Peach Council Annual
   288 Convention. South Carolina. pp. 59-68.
- Romero, C. Pedryc, A. Munoz, V. Lla'cer, G. and Badenes, M.L. 2003. Genetic diversity of different apricot geographical groups determined by SSR markers. *Genome*. 46: 244-252.
- Rout, G.R. and Das, G. 2002. An assessment of genetic integrity of micropropagated plants of *Plumbago zeylanica* by RAPD markers. *Biol Plant*. 45(1):27-32.
- Ruzic. Cerovic, R. and Vujovic, T. 2010. Establishment of aseptic culture *in vitro* for new vegetative rootstocks for
   cherry, pear and plum. *Vocarstvo* 44(169-170): 35-41.
- Srivastava, S. Jain, R. Gupta, P.S. and Singh, J. 2005. Analysis of genetic fidelity in micropropagated plants of
   sugarcane using SSR-SSCP assay. *Ind J Genet Plant Breed*. 65(4): 327-328.
- Testolin, R. Messina, R. Lain, O. Marrazzo, M.T. Huang, W.G. and Ciprani, G. 2004. Microsatellites isolated in
   almond from an AC-repeat enriched library. *Mol Ecol Notes* 4: 459-461.
- Uematsu, C. Sasakuma, T. and Ogihara, Y. 1991. Phylogenetic relationships in the stone fruit group of *Prunus* as
   revealed by restriction fragment analysis of chloroplast DNA. *Jpn J Genet*. 66: 59-69.
- Wen, X.P. and Deng, X.X. 2005. Micropropagation of chestnut rose (*Rosa roxburghii Tratt*) and assessment of
   genetic stability in *in vitro* plants using RAPD and AFLP markers. *J Hort Sci Biotechnol.* 80(1): 54-60.
- Zotto, A. D. and Docampo, D. M. 1997. Micropropagation of rootstocks of plum cv. Marianna 2624 (*Prunus cerasifera x P. munsoniana*) and Pixy (*P. insititia L.*) of certified sanitary status. *Phyton Buenos Aires*. 60(1/2): 127-135.
- Zucchi, M.I. Arizono, H. Morais, V.A. Fungaro, M.H.P. and Vieira, M.L.C. 2002. Genetic instability of sugarcane
   plants derived from meristem cultures. *Genet Mol Biol.* 25(1): 91-95.
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#### 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 <sub>3</sub> 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	

 $CD_{0.05}$ 2.45Figures in parentheses () are the arc sine transformation of percentage. 

316	Table 2. Primers used to assess genetic stability of in vitro propagated plants of Jaspi-Pri	ınus
317	ootstock	

S.	RAPD primers used					
No	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 $5' \rightarrow 3'$	Total Number of amplified bands	Total number of segments	Size range of amplified bands in base pairs	
1	SSR-1	Forward:GTAACGCTCGCTACCACAAAA	1	24	1000-2000	
2	SSR-2	Forward:TTCTAATCTGGGCTATGGCG Reverse:GAAGTTCACACATTACGACAGGG	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	SR-5 Forward:TCGGAAACTGGTAGTATGAACAGA		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 use	d	•		•	
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	СТСТСТСТСТСТСТТ	5	120	1000-3000	
2	ISSR-B	CACACACACACACACAT	2	48	800-3000	
3	ISSR-C	тетететететете	2	72	4000-5000	
4	ISSR-D	TCTCTCTCTCTCTCTCG	2	72	700-800	
5.	ISSR-E	ACACACACACACACACG	2	96	2000-3000	
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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	GAGAGAGAGAGAGAGAGACG	2	48	600-1000
11.	ISSR-L	GACAGACAGACAGACA	1	24	800-1000
12.	ISSR-M	ACACACACACACACAC	1	24	3000-4000
13.	ISSR-O	CACACACACACACAGC	1	24	650-700
14.	ISSR-P	GAGAGAGAGAGAGAGATA	3	72	1000-3000
	TOTAL		39	1032	

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Figure 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



Primer – ISSR-M 5' ACACACACACACACAC3' No. of scorable bands-1

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