http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

OPEN ACCESS **Pakistan Journal of Biological Sciences**

ISSN 1028-8880
DOI: 10.3923/pjbs.2020.1176.1183

Research Article
Molecular Assessment of Genetic Stability Using CDDP and DNA-barcoding Assays in Long-term Micropropagated Rose Plant

[1,2]El Dessoky S. Dessoky, [1,2]Attia O. Attia, [1,2]Ismail A. Ismail, [3]Saqer S. Alotaibi and [3]Bandar S. Aljuaid

[1]Department of Biology, College of Science, Taif University, Taif, Kingdom of Saudi Arabia
[2]Department of Plant Genetic Transformation, Agricultural Genetic Engineering Research Institute, Agricultural Research Centre, 12619, Giza, Egypt
[3]Department of Biotechnology, College of Science, Taif University, Taif, Kingdom of Saudi Arabia

Abstract

Background and Objective: Roses are the world's best-known garden plants, established as ornamental plants cultivated for their blooms. Taif rose (*Rosa damascena trigintipetala*) refers to the Damascus Rose species and is regarded one of Taif Governorate's most significant financial goods, which produces an extremely fragrant commercially precious essential oil. The objective of current study was to assess the genetic stability of micropropagated Taif rose and to assess the usefulness of Conserved DNA Derived Polymorphism (CDDP) and DNA-barcoding genes such as; *rpo*C1 (chloroplast gene RNA polymerase1) in the detection of somaclonal variation. **Materials and Methods:** Ten combinations of CDDP PCR primers were employed and the *rpo*C1 gene region was sequenced for mother plant (control) and micropropagated plantlets of Taif rose plant. **Results:** Based on CDDP data, phylogenetic divergence indicated that the distinct specimens of Taif rose micro-propagated plantlets and control were genetically differentiated by a difference of 1% of genetic dissimilarity. Phylogenetic tree which developed using rpoC1 DNA showed that rpoC1 DNA sequencing discovered a genetic difference between the control and micro-propagated plantlets of Taif rose. **Conclusion:** Furthermore, CDDP and DNA barcoding using *rpo*C1 gene have demonstrated their usefulness in investigating the genetic history of *Rosa* species and their ability to explore genetic mutation.

Key words: Taif rose, Conserved DNA Derived Polymorphism (CDDP), chloroplast genes RNA polymerase (*rpo*C1), DNA-barcoding, genetic stability, somaclonal variation, micro-propagation

Citation: El Dessoky S. Dessoky, Attia O. Attia, Ismail A. Ismail, Saqer S. Alotaibi and Bandar S. Aljuaid, 2020. Molecular assessment of genetic stability using CDDP and DNA-barcoding assays in long-term micropropagated rose plant. Pak. J. Biol. Sci., 23: 1176-1183.

Corresponding Author: El Dessoky S. Dessoky, Department of Biology, College of Science, Taif University, Taif, Kingdom of Saudi Arabia

Copyright: © 2020 El Dessoky S. Dessoky *et al.* This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Roses are the world's best-known garden plants, established as ornamental plants cultivated for their blooms used as cut flowers, potted plants and garden plants and regarded to be the most significant crops in the floriculture sector[1,2]. The *Rosa* genus is a part of the Rosaceae family, which has categorized more than 150 species[3]. More than 20,000 business cultivars are founded jointly on only eight wild species[4]. Taif rose, *R. damascena trigintipetala* refers to the Damascus Rose species and is regarded one of Taif Governorate's most significant financial goods, Saudi Arabia, which produces an extremely fragrant commercially precious essential oil. Taif rose plays a significant part in the domestic economy because of their high importance in the export of their oil to the Arab countries, medicinal use, ornamentation and perfume production[5,6]. Al-Taif rose products, besides rose oil are rose humidity and drained petals used in the medication, fragrance and make-up industries[7]. The seeds, stem cutting, grafting, budding, cutting-grafting, cutting-budding, root grafting and tissue culture are different methods to propagate rose species[8,9].

Tissue culture is the aseptic culture of whole plants, organs, tissues or single cells, *in vitro*, under maintained nutritional and environmental conditions for the production of plant clones. These conditions include a suitable supply of nutrients, a medium pH, a suitable temperature and a suitable gaseous and liquid environment. The resulting clones are true to the selected genotype[10]. One way of expanding clonal selection could be by inducing somaclonal distinctions varying from easy-to-detect deviations, particularly morphological characteristics, to subtle deviations in intensity, bunch, fruit dimensions and chemical content[11]. Generating true-to-type plant products in a rootstock micropropagation scheme is of excellent significance, so soma-clonal changes of any kind can multiply very quickly if caused and contribute to the loss of the primary characteristics of parent root stocks[12].

Moreover, this genetic instability may present a risk associated with the application of *in vitro* growing techniques for the processing and retention of germplasms[13]. When plant tissue is cultivated *in vitro*, many of the regenerated plantlets no longer are available to be clonal copies of their donor genotype, probably due to epigenetic changes. The wider usefulness of any micro-propagation system can be limited due to cryptic genetic changes and the development of soma-clones[14].

Molecular marker assays is one of the valuable genetic study tools, using PCR molecular markers to target genomic regions of plants. The PCR pieces may infer the plant species explored genetic diversity and population structure[15]. In addition, sophisticated analysis of molecular markers could be used to correlate PCR markers with the plant gene network, offering plant scientists greater control over their genetic assets[16,17]. Different molecular markers have been used to study the somaclonal variation in micropropagated rose such as; RAPD analysis[18] and Inter Simple Sequence Repeat (ISSR)[19].

Conserved DNA-Derived Polymorphism (CDDP) is a process for the production of plant DNA markers for protein sequences of preserved amino acids. This assay is suggested to be used in conjunction with or as a substitute for other functionally simple dominant indicator indicator methods such as focused quantitative loci mapping[20]. The CDDP was being used to evaluate genetic diversity in chickpea[21] and date palm[22].

DNA barcoding involved the sequencing of a standard DNA region as a technique of species identification[23]. In the study of various plant species, several plastid genome regions such as; *mat*K, *rbc*L, *rop*C1, *rpo*B and *trn*H-*psb*A were commonly evaluated. The plastid gene *rop*C1 has been applied to study the diversity of *Calluna*[24], *Gongora*[25] and *Apocynaceae*[26] species.

Current study has been performed to assess the genetic stability of micropropagated Taif rose plantlets. In particular, to assess the impact of protocol and long-term *in vitro* effects on induction of somaclonal variation in Taif rose and to assess the usefulness of CDDP and DNA-barcoding genes such as; *rpo*C1 in the detection of somaclonal variation.

MATERIALS AND METHODS

Study area: The study was carried out at the labs of Deanship of Scientific Research, Taif University, KSA and Plant Genetic Transformation Department, Agricultural Genetic Engineering Research Institute, Agricultural Research Centre, Giza, Egypt, during the period of March, 2017 to June, 2019.

DNA isolation The total genomic DNA was isolated from the mother plant (control) and micropropagated plantlets (6-month and 12-month) of Taif rose plant (*R. damascene trigintipetala*) by Alizadeh and Singh[12] techniques. Using DNAeasy Plant Mini Kit (Qiagen, Santa Clarita, CA) as instructed by the supplier, one control sample and 10 samples of micropropagated plantlets were used to extract DNA from 5 g tissues. The DNA quantity was evaluated by loading 2 µL of the extracted DNA into 1% agarose gel versus 10 µL of a DNA size marker (Lambda DNA Hind III digest Phi×174/Hae III digest).

Table 1: Different forward (F) and reverse (R) sequences for the PCR primers used in this study

PN	Forward	Reverse
CDDP-3	GCCCTCGTASGTSGT	GCCCTCGTASGTSGT
CDDP-4	GCASGTGTGCTCGCC	GCASGTGTGCTCGCC
CDDP-5	TGSTGSATGCTCCCG	TGSTGSATGCTCCCG
CDDP-6	CCGCTCGTGTGSACG	CCGCTCGTGTGSACG
CDDP-7	GGCAAGGGCTGCCGC	GGCAAGGGCTGCCGC
CDDP-8	GGCAAGGGCTGCCGG	GGCAAGGGCTGCCGG
CDDP-10	GCSGAGATCCGSGACCC	GCSGAGATCCGSGACCC
CDDP-11	TGGCTSGGCACSTTCGA	TGGCTSGGCACSTTCGA
CDDP-12	AAGGGSAAGCTSCCSAAG	AAGGGSAAGCTSCCSAAG
CDDP-16	ATGGGCCGSGGCAAGGTGG	ATGGGCCGSGGCAAGGTGG
*rpo*C1	GTGGATACACTTCTTGATAATGG	TGAGAAAACATAAGTAAACGGGC

PCR analysis: Ten combinations of CDDP primers and one *rpo*C1 gene region have been used in current study (Table 1). For CDDP and *rpo*C1 gene, the PCR reaction material and program amplification cycles were performed according to Collard and Mackill[20], Phong *et al.*[27]. The ultimate products of PCR were placed at 4°C. Agarose gel (8%) was used to separate PCR fragments stained with an ethidium bromide and recorded by using the Gel Doc XR technology (Bio-Rad, Hercules, CA, USA).

Bioinformatics analyses: The National Center for Biotechnology Information, Basic Local Alignment Search Tool nucleotide (NCBI BLASTn) software was used for studying orthological genes in different species by Altschul *et al.*[28] downloading similar sequences for *rpo*C1 of *R. damascena* genes from different species. These sequences have been edited using PERL scripts[29], leaving for each species only one representative gene copy. The alignment and phylogenetic analysis of orthological genes were carried out by using the ClustalW tool[30]. The phylogenetic trees representing gene interactions were built using the online tool of ITOL[31]. Online tool ClustVis was used to illustrate the outcomes of gene diversification assessment using sequence alignment similarity matrices[32]. Additionally, scored as present (1) or absent (0), the PCR fragments were used for the similarity matrix coefficients between distinct samples for phylogenetic dendrogram building.

RESULTS

PCR analysis: In this study, ten CDDP primer combinations revealed a total number of bands 117 with a mean of 11.7 bands per combination, where all combinations generated scorable PCR fragments (Fig. 1). The primer combination CDDP-14 produced the minimum number of PCR bands (7), while CDDP-2 generated the maximum (15). About 15 polymorphic bands have been collected by using CDDP primers, of which CDDP-1, CDDP-5, CDDP-12 and CDDP-14, CDDP-18 produced one band and CDDP-2 produced 3 bands, with a polymorphism percentage of 7, 8, 8, 14 and 20%, respectively (Table 2). Additionally, through CDDP assay, it could not retrieve any unique bands that are present in control and absent in Taif rose plantlets samples. Use of CDDP assay to characterize chickpea genetic variation. Ten primers generated a total of 151 polymorphic bands out of which 141, with an average of 14.1 bands per primers varying from 9 to 17.

Table 2: Genetic stability of 10 in long-term Taif rose micropropagation compared to mother plants via CDDP analysis

PN	TB	MB	PB	PP (%)
CDDP-1	14	13	1	0.07
CDDP-2	15	12	3	0.20
CDDP-4	11	11	0	0.00
CDDP-5	12	11	1	0.08
CDDP-6	10	8	2	0.20
CDDP-11	14	12	2	0.14
CDDP-12	12	11	1	0.08
CDDP-14	7	6	1	0.14
CDDP-18	10	8	2	0.20
CDDP-19	12	10	2	0.17
Total	117	102	15	12.8

PN: Primer name, TB: Total number of bands, MB: Monomorphic bands count, PB: Polymorphic bands count, PP: Polymorphism (%)

The phylogenetic divergence based on CDDP results achieved in the present research revealed that the different samples, micropropagated plantlets and the control of Taif rose plant, were genetically distinguished at a distinction of 1% of genetic dissimilarity. Only samples 1 and 2, 6 and 7 have been clustered in one group at similarity of 99.99 and 99.98%, respectively, while other samples were highly diverged and did not clustered in one group.

Bioinformatics analyses: Moreover, the control illustrated more genetic similarity with samples 1 and 2 compared to other Taif rose micropropagated plantlets samples (Fig. 2). The similarity matrix heat map by using CDDP showed that the

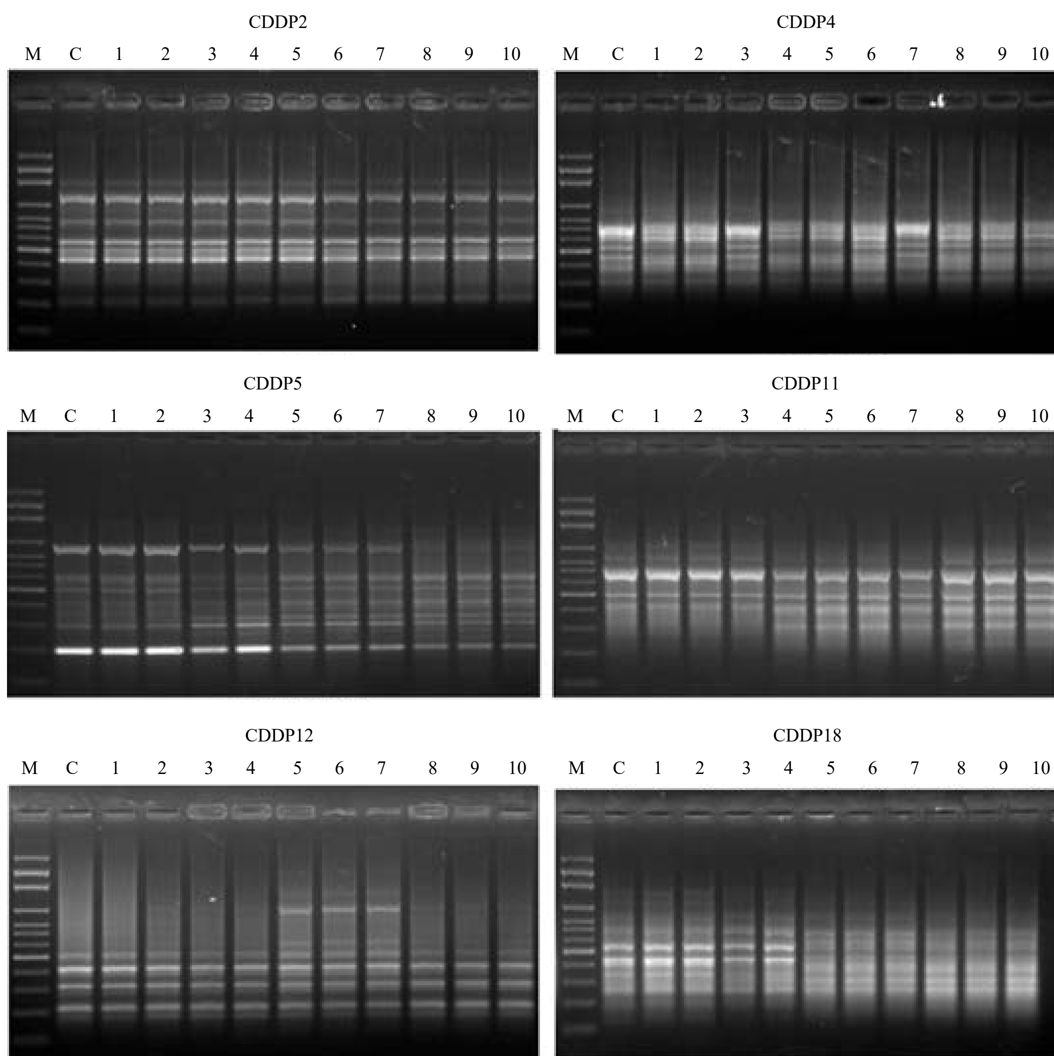

Fig. 1: CDDP PCR profile of some selected primers combinations for the control and the 10 micropropagated plantlets samples of Taif rose

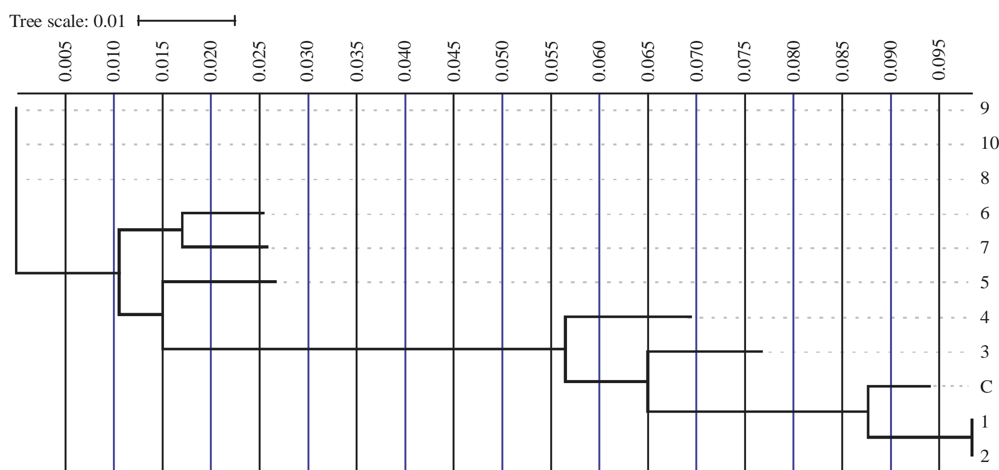

Fig. 2: Phylogenetic relationship between different Taif rose micropropagated plantlets and control depending on CDDP binary data

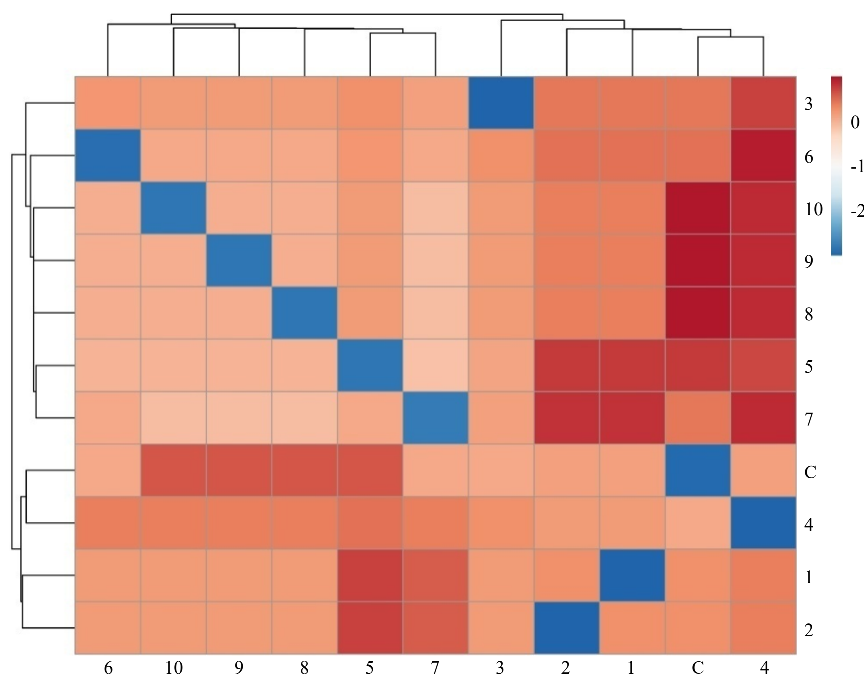

Fig. 3: Similarity matrix heat map of different Taif rose micropropagated plantlets samples and control (C) using CDDP binary data

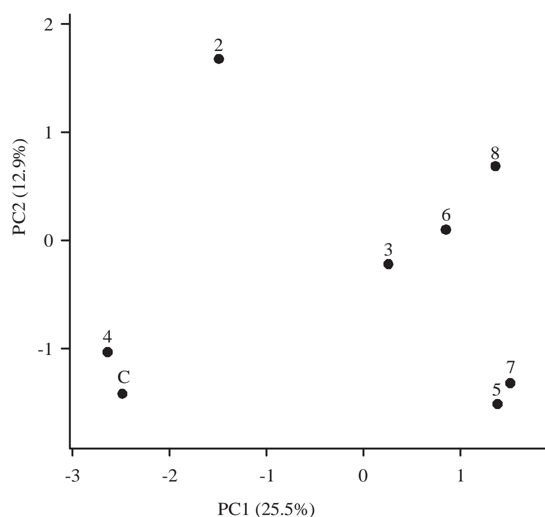

Fig. 4: PCA population structure for Taif rose micropropagated plantlets samples and control (C) based on CDDP binary data

control sample is extremely divergent with some Taif rose micro-propagated plantlets, with the exception of samples 4, 2 and 1 having a small genetic distinction rate (Fig. 3). The assessment of the PCA verified the predication of the prior data analysis on the composition of Taif rose samples. In addition, the segregation of the 4 and control samples constructions became more condensed while the other plantlets samples were spread structurally across the region (Fig. 4, 5). Different molecular markers were used in an *in vitro* propagated rose to explore the somaclonal variation. Differences in polymorphic DNA were noted through the RAPD assessment between the initial and regenerated crops. ISSR and RAPD markers have been used to evaluate genetic fidelity of *Rosa* hybrida plantlets cultivated *in vitro*. Between the explant source and all the micropropagated plantlets, a homogeneous amplification profile was found. The outcome showed the tissue culture fidelity in cultivating *Rosa hybrid* plantlets and corroborated the hypothesis that axillary multiplication is the safest way to multiply true to crops without somaclonal variation (Fig. 5).

DISCUSSION

The genetic diversity assessment of bittersweet germplasm using CDDP revealed restricted genetic variation among different species and proved the ability of CDDP with further research and selection of specimens across a broader global distribution range for the genetic enhancement of solanaceous crop gene pools[33]. The Principal Component Analysis (PCA) could be used to deduce population structure by using information from molecular makers[34].

Previous research by Malik *et al.*[35] reported that the using of barcoding markers to authenticate chosen endangered

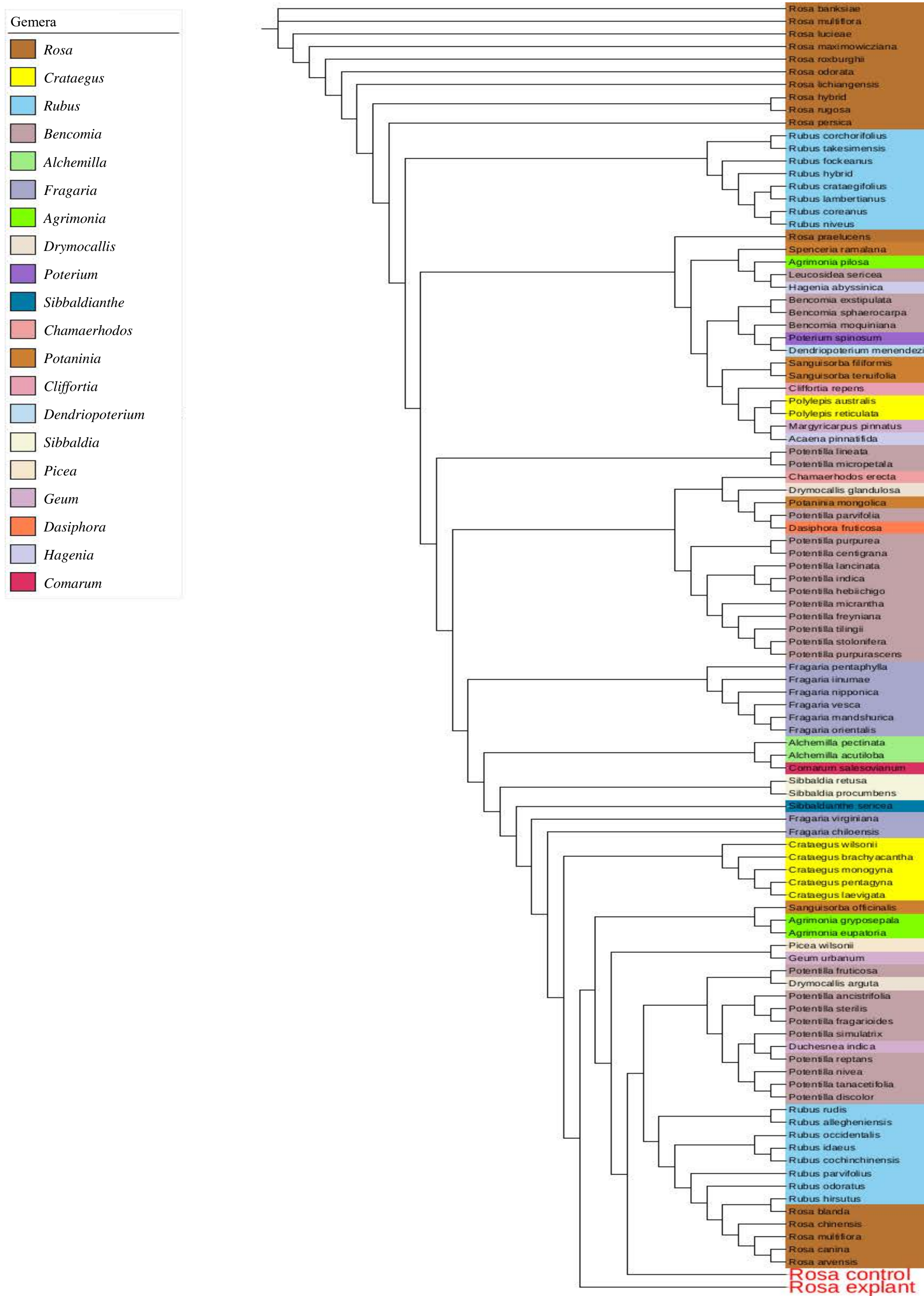

Fig. 5: Phylogenetic tree analysis comparing Taif rose *rpoC1* gene of both the control and explant with 100 plant species

medicinal plants traded in Indian markets, the recovered samples, *rbc*L, *rpo*C1 and *mat*K sequences of 7, 11 and 7 species, respectively, were species-specific. The sequences of the selected loci from the medicinal samples of these species were hard to obtain due to amplification or sequencing error. Additionally, the phylogenetic classification of *Gongora* species (Orchidaceae) based on DNA Barcode regions suggested that *rpo*B and *rpo*C1 are helpful indicators for evolutionary assessment and that trnH-psbA could elucidate distinctions between strongly associated species[25].

In the current study, the *rpo*C1 gene for chloroplast was used to infer the distinction in genetic similarity induced by the propagation of Taif rose tissue culture. *Rpo*C1 gene sequenced both the control and one micropropagated plantlets sample. The produced DNA sequences were used to assess the feasible impact on Taif rose variety of tissue culture diversity. *Rpo*C1 gene of the control and micropropagated plantlets of Taif rose plant were compared to 100 plant species recovered by the online tool of NCBI blastn. Multiple sequence alignment of the phylogenetic tree developed using *rpo*C1 genes disclosed that *rpo*C1 gene sequencing found a low genetic distinction between Taif rose control and micropropagated plantles samples. This could be due to the rapid mutation impact on chloroplast genes of the tissue culture development process, or the elevated mutation rate of *rpo*C1 compared to other genes. The *rpo*C1 successfully allocated both Taif rose samples (control and micropropagated plantlets) to a cluster where various species of *Rosa* such as; *R. blanda, R. chinensis, R. multifiora, R. canina* and *R. arvensis* were placed together. This might infer this gene usefulness in identifying wild and cultivated species of *Rosa*. In another sense, *rpo*C1 was able to distinguish between distinct genera of plants, which could infer power in identifying their species (Fig. 3). In addition, the sequence alignment of the *rpo*C1 gene of various species of plants infer low conserved region that could justify the strong species identification recorded using this gene (Fig. 4). One can recommend that further studies should be carried out at the level of physiological and horticultural characters of the micropropagated plants, which could be reflect the possible changes in the genome of the micropropagated plants

CONCLUSION

Due to the high genetic similarity identified by CDDP assay and *rpo*C1 gene sequencing, it could be deduced that micro-propagation through tissue culture has a small effect on the genetic content of Taif rose plant. The result indicated the clonal fidelity of the tissue culture raised *R. damascena* plantlets and corroborated the assumption that axillary multiplication is the safest way to multiply true to type plants with negligible rate of somaclonal variation. In addition, CDDP and DNA-barcoding (*rpo*C1) assays have shown their usefulness in investigating the genetic history of *Rosa* species and have shown their potential for exploring genetic diversity and genetic mutation.

SIGNIFICANCE STATEMENT

This study paid an attention to the importance of determination of genetic variation of micropropagated plants that can be beneficial for their stability. In other words, no qualitative and/or quantitative changes in the morphology or productivity of micropropagated plants could be addressed. This study will help the researcher to well understand the possibility of genome genetic variation in the micropropagated plants by using more advanced molecular tools.

REFERENCES

1. Babaei, A., S. Tabaei-Aghdaei, M. Khosh-Khui, R. Omidbaigi, M. Naghavi, G.D. Esselink and M.J.M. Smulders, 2007. Microsatellite analysis of Damask rose (*Rosa damascena* Mill.) accessions from various regions in Iran reveals multiple genotypes. BMC Plant Biol., Vol. 7, No. 1. 10.1186/1471-2229-7-12
2. Nour El-Deen, A.H., Y.D. Hadeer, A.A. El-Ghamdi and B.N. Samra, 2015. Evaluating the pathogenicity of nematodes infecting roses at Taif governorate, KSA. Res. J. Pharm. Biol. Chem. Sci., 6: 1562-1567.
3. Abdel-Hameed, E.S., S.A. Bazaid and M.S. Salman, 2013. Characterization of the phytochemical constituents of taif rose and its antioxidant and anticancer activities. BioMed Res. Int., Vol. 2013. 10.1155/2013/345465
4. Kim, S.H. and J.H. Lieth, 2003. A coupled model of photosynthesis, stomatal conductance and transpiration for a rose leaf (*Rosa hybrida* L.). Ann. Bot., 91: 771-781.
5. El-Assal, S.E., M.A. El-Awady, A. El-Tarras and G. Shehab, 2014. Assessing the genetic relationship of taif rose with some rose genotypes (*Rosa* sp.) based on random amplified polymorphic DNA, inter simple sequence repeat and simple sequence repeat markers. Am. J. Biochem. Biotechnol., 10: 88-98.
6. Jalali-Heravi, M., H. Parastar and H. Sereshti, 2008. Development of a method for analysis of Iranian damask rose oil: Combination of gas chromatography–mass spectrometry with Chemometric techniques. Anal. Chim. Acta, 623: 11-21.
7. Nariman, A., K. Salama and M. Abouseada, 2011. Improvement of production and quality of Al-Taif Rose via tissue culture techniques. Adv. Scholar Med., 1: 22-25.

8. Dole, J.M. and H.F. Wilkins, 1999. Floriculture: Principles and Species. Prentice Hall, Upper Saddle River, N.J., USA.
9. Attia, A.O., E.D.S. Dessoky and A.E. El-Tarras, 2012. *In vitro* propagation of *Rosa hybrida* L. cv. Al-Taif rose plant. Afr. J. Biotechnol., 11: 10888-10893.
10. Hussain, A., H. Nazir, I. Ullah and I.A. Qarshi, 2012. Plant Tissue Culture: Current Status and Opportunities. In: Recent Advances in Plant *In Vitro* Culture, Leva, A. and L. Rinaldi (Eds.)., INTECH Open Access Publisher, Rijeka, Croatia, ISBN: 978-953-51-0787-3, pp: 1-28.
11. Schellenbaum, P., V. Mohler, G. Wenzel and B. Walter, 2008. Variation in DNA methylation patterns of grapevine somaclones (*Vitis vinifera* L.). BMC Plant Biol., Vol. 8, No. 1. 10.1186/1471-2229-8-78
12. Alizadeh, M. and S.K. Singh, 2009. Molecular assessment of clonal fidelity in micropropagated grape (*Vitis* spp.) rootstock genotypes using RAPD and ISSR markers. Iran. J. Biotechnol., 7: 37-44.
13. Ray, T., I. Dutta, P. Saha, S. Das and S.C. Roy, 2006. Genetic stability of three economically important micropropagated banana (*Musa* spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. Plant Cell Tissue Org. Cult., 85: 11-21.
14. Rani, V. and S.N. Raina, 2000. Genetic fidelity of organized meristem-derived micropropagated plants: A critical reappraisal. *In vitro* Cell. Dev. Biol.-Plant, 36: 319-330.
15. Ibrahim, S.D., S.S. Adawy, M.A.M. Atia, A.M. Alsamman and M.M. Mokhtar, 2016. Genetic diversity, variety identification and gene detection in some Egyptian grape varieties by SSR and SCoT markers. Plant Omics J., 9: 311-318.
16. Alsamman, A.M., S.D. Ibrahim and A. Hamwieh, 2019. KASPspoon: An *in vitro* and *in silico* PCR analysis tool for high-throughput SNP genotyping. Bioinformatics, 35: 3187-3190.
17. Habib, P.T., A.M. Alsamman and A. Hamwieh, 2019. BioAnalyzer: Bioinformatic software of routinely used tools for analysis of genomic data. Adv. Biosci. Biotechnol., 10: 33-41.
18. Asano, G. and S. Tanimoto, 2011. Plant regeneration from embryogenic calli derived from immature seeds in miniature rose cultivar. 'Shortcake'; somaclonal variation, cytological study and RAPD analysis. Plant Biotechnol., 19: 271-275.
19. Senapati, S.K., S. Aparajita and G.R. Rout, 2012. An assessment of genetic fidelity of *in vitro* grown plantlets of rose (*Rosa hybrid*) through molecular markers. Afr. J. Biotechnol., 11: 16532-16538.
20. Collard, B.C.Y. and D.J. Mackill, 2009. Conserved DNA-Derived Polymorphism (CDDP): A simple and novel method for generating DNA markers in plants. Plant Mol. Biol. Rep., 27: 558-562.
21. Hajibarat, Z., A. Saidi, Z. Hajibarat and R. Talebi, 2015. Characterization of genetic diversity in chickpea using SSR markers, start codon targeted polymorphism (SCoT) and conserved DNA-derived polymorphism (CDDP). Physiol. Mol. Biol. Plants, 21: 365-373.
22. Adawy, S.S. and M.A.M. Atia, 2014. A multidisciplinary molecular marker approaches to assess the genetic diversity in egyptian date palm. Int. J. Bio-Tech. Res., 4: 1-12.
23. CBOL Plant Working Group, 2009. A DNA barcode for land plants. Proc. Natl. Acad. Sci. USA., 106: 12794-12797.
24. Cristina-Mirela, C., P. Ioana-Gabriela, M. Caprar and C. Sicora, 2015. Evaluating the potential of a few barcode markers in identifying the species *Calluna vulgaris* (L.) Hull. J. Hortic. For. Biotechnol., 19: 57-61.
25. Guardado, C., O. Rodriguez, K. Foss, D. Ludovic and C. Ribadeneyra *et al*., 2016. Phylogenetic classification of *Gongora* species (Orchidaceae) based on DNA barcode regions matK, rpoB, rpoC1 and trn-H-psbA. Biochem. Mol. Biol., Vol. 30. 10.1096/fasebj.30.1_supplement.572.3
26. Selvaraj, D., R.K. Sarma, D. Shanmughanandhan, R. Srinivasan and S. Ramalingam, 2015. Evaluation of DNA barcode candidates for the discrimination of the large plant family *Apocynaceae*. Plant Syst. Evol., 301: 1263-1273.
27. Phong, D.T., V.T.T. Hien and T.T. Lieu, 2018. Nucleotide diversity of 15 conifer species in vietnam's central highlands based on the analysis of its, trnH-psbA, matK, trnL AND rpoC1 gene regions. Vietnam J. Sci. Technol., 56: 47-63.
28. Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller and D.J. Lipman, 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucl. Acids Res., 25: 3389-3402.
29. Wall, L., 1994. The perl programming language. The perl programming language
30. Thompson, J.D., D.G. Higgins and T.J. Gibson, 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res., 22: 4673-4680.
31. Letunic, I. and P. Bork, 2006. Interactive tree of life (iTOL): An online tool for phylogenetic tree display and annotation. Bioinformatics, 23: 127-128.
32. Letunic, I. and P. Bork, 2016. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. Nucleic Acids Res, 44: W242-W245.
33. Poczai, P., I. Varga, N.E. Bell and J. Hyvönen, 2011. Genetic diversity assessment of bittersweet (*Solanum dulcamara*, Solanaceae) germplasm using conserved DNA-derived polymorphism and intron-targeting markers. Ann. Applied Biol., 159: 141-153.
34. Lee, C., A. Abdool and C.H. Huang, 2009. PCA-based population structure inference with generic clustering algorithms. BMC Bioinformatics, Vol. 10. 10.1186/1471-2105-10-S1-S73
35. Malik, S., A. Priya and S.B. Babbar, 2018. Employing barcoding markers to authenticate selected endangered medicinal plants traded in Indian markets. Physiol. Mol. Biol. Plants, 25: 327-337.