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RESEARCH ARTICLE

Effect of different combinations of plant growth regulators and assessment of clonal fidelity among *in vitro* raised micro-shoots of pomegranate (*Punica granatum* L.) cv. Bhagwa

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Abstract

The role of sodium nitroprusside (SNP) in plant development and growth is varied. However, there is currently no reported use of SNP in single or combined treatments for *in vitro* propagation of pomegranates. This study elaborates the role of SNP combined with different plant growth regulators (PGRs) like kinetin (Kn) and indole acetic acid (IAA) as supplementary in Murashige and Skoog (MS) media for *in vitro* propagation of pomegranate cv. Bhagwa using nodal segments as explants. The results revealed that treatment T_9 (1.5 mg/LSNP + 1.5 mg/LKn) resulted in the earliest shoot induction (25.03 days), the highest shoot proliferation (86.67 %) and the maximum shoot length (3.10 cm). However, treatment T_8 (1.5 mg/LSNP + 1.0 mg/LKn) produced the highest number of shoots/explant (2.60). In regards to *in vitro* rooting, the treatment fortified with T_{14} (2.0 mg/LIAA + 0.1 mg/LSNP) yielded the highest rooting frequency (100 %) with the maximum number of roots (4.77), while the treatment T_{15} (2.0 mg/LIAA + 0.2 mg/LSNP) exhibited earlier root initiation (13.17 days). The clonal fidelity analysis using ISSR markers revealed that the micro-propagated shoots were genetically uniform, with 88.89 % showing uniformity and a low level of somaclonal variation at 11.11 %.

Keywords

micropropagation; ISSR marker; pomegranate; plant growth regulators; clonal fidelity; quality maintenance; *in vitro* culture

Introduction

Punica granatum L., commonly known as pomegranate, holds significant economic value as a commercial fruit crop in tropical and subtropical regions across the globe. *P. granatum* L., a member of the Lythraceae family, is a fruit with a long history of consumption. The pomegranate, originally indigenous to Iran, has become a globally cultivated fruit, with significant production in countries such as India, Turkey, China, the United States, Egypt, Tunisia, Morocco, Spain and South Africa (1). It is considered to be one of the earliest known edible fruits. Furthermore, the fruit is highly valued for its nutritious edible qualities, lucrative returns, substantial export demand, versatile adaptability, minimal irrigation water requirements and pharmaceutical applications (2). India and Iran are the leading nations in terms of exports, with a notable increase in their output on a global level (3).

The cultivation of this crop spans across an extensive area of 2.83 lakh ha in India, yielding a production of 32.71 lakh metric tonnes, as reported by the NHB Data Book 2020–21 (4).

Over the past few years, bacterial blight and wilt diseases have resulted in significant crop loss. The establishment of new plants necessitates a year-long process and is impeded by the presence of insect and pest invasions/diseases (5). Additionally, the establishment of new plants is restricted by the scarcity of mother plants. The use of conventional propagation methods does not guarantee the production of disease-free, healthy plantlets (6). The use of nodal segments for *in vitro* propagation of pomegranate was observed (7). Utilization of micropropagation techniques has experienced a substantial surge in extensive propagation over the last few decades. The use of tissue culture as a propagation technique offers several advantages over traditional methods. These benefits include consistent quality, freedom from seasonal limitations and the ability to rapidly produce large quantities of disease-free plant material (8, 9). In fact, *in vitro* culture is currently the only mass propagation method capable of producing healthy plants within a short timeframe (10).

PGRs are essential for the successful implementation of micropropagation. In the pomegranate, the function of PGRs has been improved by a number of researchers (11-14). The most critical components for successful plant regeneration are plant growth regulators. Cytokinins are essential for the development of meristematic centers, which induce cell division and result in the production of organs, primarily shoots, in tissue culture (12, 14). Likewise, SNP, which serves as the main provider of nitric oxide (NO), has been classified as a phytohormone with various effects on plant growth and development. SNPs can affect plant morphogenesis, reduction of seed dormancy, promotion of lateral root growth, facilitation of germination, stimulation of shoot regeneration, facilitation of root formation and regulation of senescence (15, 16). SNPs in the medium increased adventitious root production, improved callus formation and multiplied shoot regeneration in *Gymnema sylvestre* (17). However, there are no known reports where SNP has been used as a single PGR or in combination with other PGRs for *in vitro* propagation of pomegranate. Sodium nitroprusside has also been reported in conjunction with other PGRs in the *in vitro* propagation of diverse crops (18, 19). In order to maximize *in vitro* efficiency in pomegranate, we adjusted the combination of SNPs with other PGRs in the current study.

Clonal fidelity of the micropropagated plants refers to the genetically identical plants called clones and it is essential to ensure that the propagated plants retain the same genetic characteristics as the original parent plant. The potential for somaclonal diversity among the subclones of prospective lines, however, is a significant issue with *in vitro* culture (20). The changes in micro-plants at the cellular level and at the ploidy level may be caused by genetic modifications in the structures of the chromosome or molecular variations like specific DNA mutations (21). Among the molecular markers, inter-simple sequence repeat (ISSR) markers are widely used in clonal fidelity analysis due to their ability to produce highly variable and reproducible bands across a range of plant species. The use of longer primers, which enable higher annealing temperatures, makes the ISSR very reproducible (22). ISSR markers are based on the amplification of DNA fragments between simple sequences repeat using primers anchored at these repeats. ISSR markers are highly polymorphic and they can detect differences in the DNA sequence even among closely related genotypes. ISSR markers have been used in various applications, including genetic diversity analysis, phylogenetic studies and clonal fidelity analysis in plant tissue culture (22, 23). In clonal fidelity analysis, ISSR markers are used to compare the DNA profiles of the micro-propagated plants to those of the original parent plant (23). Any differences observed in the ISSR profiles may indicate somaclonal variation, which refers to genetic changes that occur during tissue culture. Clonal fidelity analysis using ISSR markers is a valuable tool for quality control in micropropagation (24). It allows for the early detection of any genetic variations that may affect the quality and yield of the micro-propagated plants (22). This analysis can also provide insights into the stability and adaptability of the micro-propagated plants in different environments. With the aforementioned information in mind, the current work was conducted to optimise the SNP concentration with additional PGRs that had not been performed previously in pomegranate and examine the clonal stability of micro-plants with the help of ISSR markers.

Materials and Methods

Plant materials and surface sterilization of explant

A stem cutting of the pomegranate cultivar Bhagwa, about 5 cm long and 1–2 months old, was taken from the Uttar Pradesh region. The Horticulture Research Centre (HRC) of Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, U.P., India, established a block for donor plants. The maternal plants underwent weekly treatment with Tebuconazole 50 % + Trifloxystrobin 25 % w/w, a systemic fungicide manufactured by Bayer Crop Science, at a concentration of 2 g/L prior to the commencement of the experimental cutting procedures. The young cuttings, which were in good health and free from disease, underwent a 20 min rinse under tap water. Subsequently, they were subjected to a 10 min treatment with Teepol at a concentration of 0.1 %, followed by a 20 min treatment with Bavistin at the same concentration. The cuttings were then subjected to multiple rinses with deionized water and exposed to UV radiation for 20 min under laminar air flow. The explants were subjected to treatment with a 0.1 % $HgCl_2$ solution for duration of 1–2 min under a cabinet. This was followed by treatment with 70 % ethyl alcohol for 1 min and a final step of four rinses with sterilised double-distilled water to ensure sterilisation. The nodal segments, measuring approximately 5–6 mm, were obtained by trimming the explants to eliminate the edges that were typically damaged during the disinfection procedure after surface sterilization.

Media preparation

The current work utilised the basal Murashige and Skoog, 1962 media supplemented with several plant growth regulators (PGRs) for the purpose of shoot proliferation and root induction (25). The experiments involved the supplementation of various combinations of SNP, Kn and IAA. A concentration of 200 mg/Lof activated charcoal (AC) as an additive was added in all of the combinations. C source-2.5 % sucrose was incorporated and the media was solidified using Cleri Gel at a concentration of 7 g/L. The pH of the solution was kept in the range of 5.6 to 5.8. The culture media was transferred into 25 × 150 mm culture tubes and sealed with polypropylene closures. These tubes were then autoclaved at a pressure of 1.05 kg cm^{-2} for duration of 20 min. The thoroughly surface-sterilized explants were cultured on MS basal media, fortified with various concentrations and combinations of plant growth regulators. After inoculation, the cultures were transferred to a growth chamber.

Culture multiplication and regeneration

The establishment of *in vitro* cultures was conducted using test tubes measuring 20 mm by 170 mm and containing 15 mL of MS culture media. Varying concentrations of SNP and Kn were used for *in vitro* regeneration of shoots, and SNP with IAA in various concentrations was used for *in vitro* rooting of shoots. The experiment involved testing these compounds individually and in combination with each other. Healthy nodal segments of shoots measuring 20–25 mm were used as explants for *in vitro* conditions. A total of nine combinations of SNP with Kn were evaluated for their impact on shooting, while 16 different combinations of IAA and SNP were used as rooting parameters (Table1). The duration between inoculation and the initial emergence of a bud from the explants was measured in days to determine the time required for shoot initiation. The shoot regeneration percentage was calculated by dividing the number of explants that produced shoots by the total number of explants and then multiplying the result by 100. In addition, the numbers of shoots per explant were manually recorded and the length of the longest shoot was measured in cm by a measuring scale after 5 weeks. The well-developed shoots were subjected to sub -culturing procedures using their respective growth media. These shoots were then transplanted onto a medium containing MS supplemented with varying concentrations of IAA (0.5–1.5 mg/L) and SNP (0.05–0.3 mg/L). Rooting percentages were determined by dividing the number of shoots that developed roots by the total number of shoots and then multiplying the result by 100. The duration of root induction and the quantity of roots were assessed.

DNA extraction

Genomic DNA was extracted from leaves obtained from ten randomly selected *in vitro* propagated plants and the maternal plant. The DNA isolation process utilized the CTAB (Cetyltrimethylammonium bromide) method, with minor modifications from the originally outlined methodology (26). The assessment of the purity of the isolated DNA samples was conducted through electrophoresis on a 0.8 % agarose gel (Hi media, Mumbai) and using a spectrophotometer (Perkin-Elmer Lambda 35 instrument). Subsequent to the collection of DNA samples, an evaluation was conducted to determine the degree of genetic uniformity through the utilisation of PCR-based ISSR markers (Table 2).

Table 1. Treatment details of *in vitro* shoot and root regeneration in pomegranate plant.

Treatment of different concentration of SNP and KN for *in vitro* shoot regeneration	
Notation	**Treatment**
T_1	SNP 0.5 mg/L+ KN 0.5 mg/L
T_2	SNP 0.5 mg/L+ KN 1.0 mg/L
T_3	SNP 0.5 mg/L+ KN 1.5 mg/L
T_4	SNP 1.0 mg/L+ KN 0.5 mg/L
T_5	SNP 1.0 mg/L+ KN 1.0 mg/L
T_6	SNP 1.0 mg/L+ KN 1.5 mg/L
T_7	SNP 1.5 mg/L+ KN 0.5 mg/L
T_8	SNP 1.5 mg/L+ KN 1.0 mg/L
T_9	SNP 1.5 mg/L+ KN 1.5 mg/L
Treatment of different concentration of IAA and SNP for *in vitro* root regeneration	
T_1	IAA 0.5 mg/L+ SNP 0.05 mg/L
T_2	IAA 0.5 mg/L+ SNP 0.1 mg/L
T_3	IAA 0.5 mg/L+ SNP 0.2 mg/L
T_4	IAA 0.5 mg/L+ SNP 0.3 mg/L
T_5	IAA 1.0 mg/L+ SNP 0.05 mg/L
T_6	IAA 1.0 mg/L+ SNP 0.1 mg/L
T_7	IAA 1.0 mg/L+ SNP 0.2 mg/L
T_8	IAA 1.0 mg/L+ SNP 0.3 mg/L
T_9	IAA 1.5 mg/L+ SNP 0.05 mg/L
T_{10}	IAA 1.5 mg/L+ SNP 0.1 mg/L
T_{11}	IAA 1.5 mg/L+ SNP 0.2 mg/L
T_{12}	IAA 1.5 mg/L+ SNP 0.3 mg/L
T_{13}	IAA 2.0 mg/L+ SNP 0.05 mg/L
T_{14}	IAA 2.0 mg/L+ SNP 0.1 mg/L
T_{15}	IAA 2.0 mg/L+ SNP 0.2 mg/L
T_{16}	IAA 2.0 mg/L+ SNP 0.3 mg/L

Table 2. ISSR primers and their sequences used for the genetic fidelity evaluation of pomegranate plants obtained from micropropagation.

Primers name	Sequence primer
UBC831	CTCTCTCTCTCTCTCTT
UBC873	GACAGACAGACAGACA
UBC868	GAAGAAGAAGAAGAAGAA
IS7	ACGACGACGACGACGG
IS15	ACACACACACACACACT
IS25	GGATGGATGGATGGAT

Clonal fidelity analysis with ISSR markers

To evaluate the genetic consistency of both the parent plants and *in vitro* plantlets, the following procedure was implemented. Initially, a preliminary selection of ISSR

primers was conducted using 10 primers. Out of these, 6 primers that consistently produced amplified fragments, ranging from 15 to 18 nucleotide bases, were chosen for further analysis. PCR reactions were carried out in a total volume of 25 µL. A master mix comprising a 20 µL reaction mixture was prepared for the PCR amplification process. The amplification conditions included a predetermined set of reaction cycles, with the initial denaturation step running at 94 °C for 5 min; then 35 cycles of denaturation step were followed at 94 °C for 30 sec; thereafter primer annealing was performed at 55 °C for 30 sec (it is important to note that different primers may require distinct annealing temperatures) and extension at 72 °C for 30 sec. A final step called extension was performed at 72 °C for 10 min. PCR amplicon was electrophoresed on an agarose gel (3 %) containing 0.5 mg/L of ethidium bromide. The gel was visualized under a UV light trans-illuminator. Only visible and clear bands were scored at specific positions and were considered for calculating and evaluating clonal fidelity (27).

Experimental design and statistical analysis

A completely randomized block design (CRD) with 10 replications per treatment was used in this study. Each treatment was replicated in 3 sets. The duration of shoot induction, percentage of shoot regeneration, quantity of shoots and length of shoots were analysed statistically through one-way analysis of variance. Tukey's HSD significance test ($p<0.05$) was performed using SPSS software (version 17.0) to determine significant differences among the treatments for all variables assessed.

Results and Discussion

Effect of SNP and Kinetin on in vitro shoot induction, shoot regeneration, number of shoots and shoot length of explants

The 2 types of plant growth regulators most commonly utilized for *in vitro* cultivation of plant tissues are cytokinin and auxin (28). SNP, a widely occurring bioactive compound, produces NO, a highly reactive gas that serves as a crucial player in signal transduction within stressed plants (29). Due to its cost-effectiveness, widespread usage and consistent generation of NO, SNP is commonly employed as an NO donor. In plant tissue and organ culture, the bioactive molecule SNP stimulates the induction of multiple shoots and roots (18, 30-32).

The experimental results presented in Table 3 and Fig. 1 indicate significant effect of nutrient media and SNP, in conjunction with varying doses of Kn (0.5 mg/L to 1.5 mg/L), on the *in vitro* propagation of pomegranate. The shortest duration for shoot induction (25.03 ± 1.1a) was observed in treatment T_9 (1.5 mg/L SNP + 1.5 mg/L Kn), while the control group (41.67 ± 2.8b) had the longest duration. Additionally, treatment T_9 (1.5 mg/L SNP + 1.5 mg/L Kn) exhibited the highest shoot regeneration percentage (86.6 ± 5.7 %), followed by treatment T_6 (1.0 mg/L SNP + 1.5 mg/L Kn) with a shoot regeneration percentage of 83.33 ± 5.7 %, while the lowest shoot regeneration percentage of 33.3 ± 5.7 % was observed in the control. Treatment T_8 (1.5 mg/L SNP + 1.0 mg/L Kn) resulted in the highest number of shoots per explant (2.60 ± 0.44), followed by treatment T_9 (1.5 mg/L SNP + 1.5 mg/L Kn) with a similar outcome (2.53 ± 0.65), while the control group had the lowest number of shoots per explant (0.80 ± 0.10). The longest shoot length of 3.10 ± 0.78 cm at 35 days after initiation (DAI) was observed in treatment T_9 (1.5 mg/L SNP and 1.5 mg/L Kn), followed by 2.40 ± 0.61 cm in treatment T_8 (1.5 mg/L SNP and 1.0 mg/L Kn), while the control group had the shortest shoot length (0.70 ± 0.26 cm) at 35 DAI.

During culture growth, cells exhibited de-differentiation and re-differentiation. It is believed that SNPs interact with auxin and cytokinin, regulating cell division during the differentiation process. Similarly, kinetin has been found to have a positive impact on *in vitro* shoot multiplication in aloe vera (33). The mineral composition of explants may also contribute to variations in the effects of Kn on multiplication rate and shoot growth across different media. The higher percentage of callus induction and shoot regeneration can be attributed to the combined application of SNP, Kn and auxin (34). Achieving optimal shoot regeneration percentage requires balancing the dose and concentration of SNP. It has been reported that the regeneration capacity decreases at high doses of SNP in the medium (35). The findings of the present study indicate that sodium nitroprusside interacts with auxin and

Table 3. Effect of SNP and Kn on *in vitro* shoot establishment of pomegranate.

Treatment (SNP + Kn mg/L)	Time taken for shoot induction (4 days)	% Culture exhibiting shoot regeneration	Number of shoots/ explant	Shoot length recorded 35 DAI in (cm)
Control	41.67 ± 2.83b	33.33 ± 5.77a	0.80 ± 0.10a	0.70 ± 0.26a
T_1 (0.5 + 0.5)	25.83 ± 1.19a	70.00 ± 10.00bcd	2.23 ± 0.45abc	2.37 ± 0.64a
T_2 (0.5 + 1.0)	26.43 ± 2.46a	56.67 ± 5.77ab	1.30 ± 0.44ab	2.10 ± 0.61a
T_3 (0.5 + 1.5)	30.17 ± 1.40a	50.00 ± 0.00a	1.77 ± 0.81abc	1.77 ± 0.61a
T_4 (1.0 + 0.5)	26.00 ± 1.37a	63.33 ± 5.77abc	1.40 ± 0.36abc	2.30 ± 0.44a
T_5 (1.0 + 1.0)	28.23 ± 3.49a	56.67 ± 5.77ab	0.97 ± 0.25a	1.90 ± 0.72a
T_6 (1.0 + 1.5)	29.50 ± 2.12a	83.33 ± 5.77de	1.17 ± 0.35a	2.03 ± 0.15a
T_7 (1.5 + 0.5)	30.27 ± 3.06a	56.67 ± 5.77ab	1.00 ± 0.20a	1.57 ± 0.12a
T_8 (1.5 + 1.0)	25.20 ± 2.05a	76.67 ± 5.77code	2.60 ± 0.44c	2.40 ± 0.61a
T_9 (1.5 + 1.5)	25.03 ± 1.12a	86.67 ± 5.77e	2.53 ± 0.65bc	3.10 ± 0.78a
SE (m)	**1.14**	**3.52**	**0.244**	**0.323**

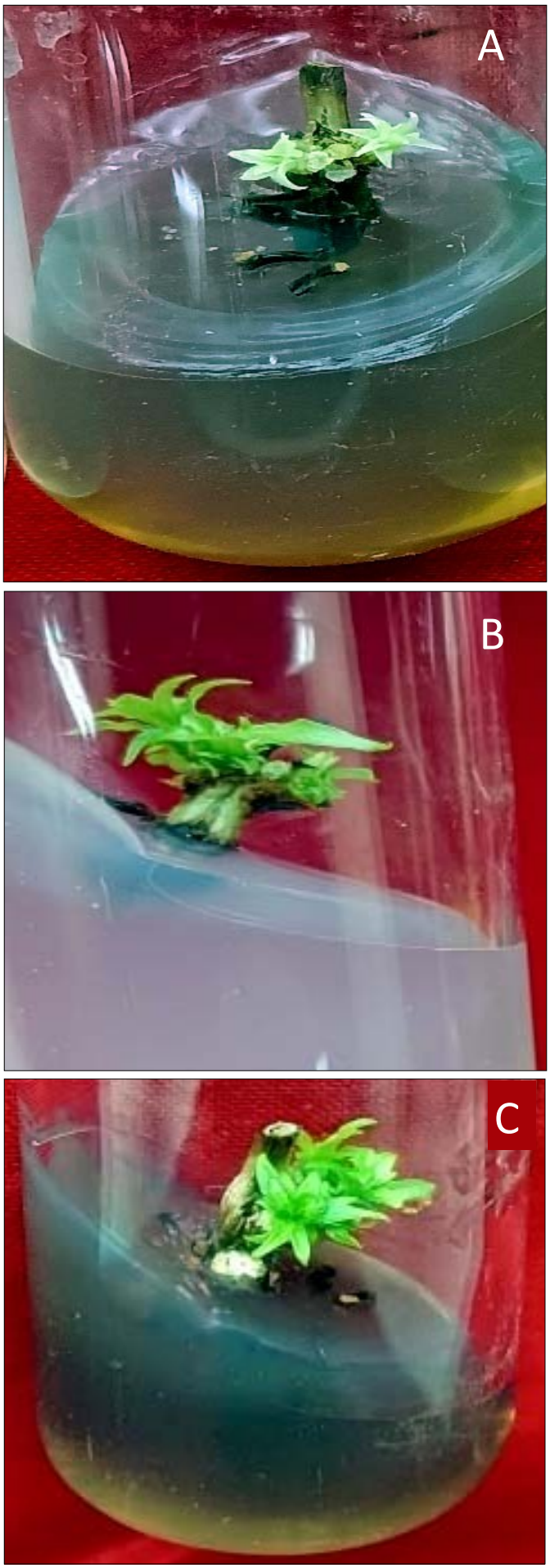

Fig. 1. *In vitro* shoot induction in pomegranate variety "Bhagwa" after 4 weeks of culture: (**A**) Control, (**B**) T_8-1.5 mg/L SNP and 1.0 mg/L Kn and (**C**) T_9-1.5 mg/L SNP and 1.5 mg/L Kn.

cytokinin during the de-differentiation and re-differentiation process during culture growth and development that regulate cell division during the differentiation process (35). Further, *in vitro* regeneration and shoot differentiation process might be improved through involvement of NO during cytokinin cell signalling. Providing cytokinin exogenously in the culture media may help to release NO during cell cultures (36). SNPs can increase membrane fluidity, affect the phospholipid bilayer, relax the cell wall, increase cell size and promote plant development. Additionally, SNP application has been shown to enhance cotton stem growth (37). An increased number of shoots was observed in marigold when SNP was used in combination with other plant growth regulators (PGRs) (38, 39). The addition of 10 % coconut water to the media, along with 15 M SNP, resulted in the highest response for *in vitro* shoot multiplication (89.3 %) in *Valeriana jatamansi* (32). Similar findings were reported in *Glycine max*, where exogenous supplementation of SNP enhanced *in vitro* responses (40). Likewise, SNP at 20.0 µM significantly promoted shoot production in *Malus hupehensis* plantlets (41). On the other hand, addition of SNP at 40.0 µM to MS medium containing BAP and NAA reduced browning and improved the cell survival of tuber explants in *Dioscorea opposite* (35). The effect of NO on caulogenesis, shoot organogenesis and rhizogenesis from hypocotyl explants of *Linum usitatissimum* reported that media supplemented with NO donors such as 5.0 µM SNP, 2.0 µM SNAP or 2.0 µM SIN-1 significantly promoted shoot differentiation (42). Similar results with a lower concentration of SNP in *Albizzia lebbeck*, where B5 basal medium containing 4.0 µM SNP stimulated caulogenesis (43).

Root regeneration percentage

In the present study, the effects of combining IAA and SNP at different concentrations in MS media for *in vitro* root were examined. The results are presented in Table 4 and Fig. 2 depicts that treatment T_{14} (2.0 mg/L IAA + 0.1 mg/L SNP) resulted in the highest rooting percentage (100.0 ± 0.0e), while higher doses of SNP and IAA decreased the quantity of *in vitro* roots. The control had the lowest root percentage (41.6 ± 2.8b). These findings suggest that very low concentrations of SNP and IAA have minimal impact on the number of *in vitro* roots, but the combined application of IAA and SNP promotes root formation compared to the control. The control exhibited the longest time for root initiation (33.3 ± 5.7 days), while treatment consisting of T_{15} (2.0 mg/L IAA and 0.2 mg/L SNP) had the shortest time (13.1 ± 0.5 days) for root initiation. Treatment T_{14} (2.0 mg/L IAA + 0.1 mg/L SNP) resulted in the highest number of roots per explant (4.7 ± 0.7), followed by treatment T_{15} (2.0 mg/L IAA + 0.2 mg/L SNP) with 4.5 ± 0.7 roots per explant. On the other hand, control had the lowest number of roots per explant (0.8 ± 0.1).

Inducing roots on shoots created through *in vitro* multiplication procedures can be difficult under normal circumstances. To enhance *in vitro* rooting, plant shoots are generally cultured on MS media, which is fortified by various concentrations of auxins, *viz*. IAA, IBA and NAA (31,

Table 4. Effect of MS media with IAA and SNP on *in vitro* root establishment of pomegranate.

Treatment (IAA+SNP mg/L)	% Culture exhibiting root develoment	Time taken for root initiation (Days)	Number of roots/explant
Control	41.67 ± 2.83b	33.33 ± 5.77a	0.80 ± 0.10a
T_1 (0.5 + 0.05)	73.33 ± 5.77abcs	25.3 ± 1.95f	1.07 ± 0.15a
T_2 (0.5 + 0.1)	70.00 ± 10.00abc	18.97 ± 0.47e	1.13 ± 0.35a
T_3 (0.5 + 0.2)	63.33 ± 5.77a	18.77 ± 1.06e	1.53 ± 0.35ab
T_4 (0.5 + 0.3)	66.67 ± 15.28ab	18.07 ± 1.12de	2.17 ± 0.32abcs
T_5 (1.0 + 0.05)	83.33 ± 5.77abide	17.80 ± 0.61de	2.13 ± 0.35abcs
T_6 (1.0 + 0.1)	86.67 ± 5.77abide	17.20 ± 0.36code	2.60 ± 0.79bcd
T_7 (1.0 + 0.2)	90.00 ± 10.00bode	16.80 ± 0.7code	3.23 ± 0.45de
T_8 (1.0 + 0.3)	90.00 ± 0.00bode	16.17 ± 0.35bcd	3.07 ± 0.15code
T_9 (1.5 + 0.05)	83.33 ± 15.28abide	15.13 ± 0.90abc	1.87 ± 0.21abc
T_{10} (1.5 + 0.1)	93.33 ± 11.55code	15.23 ± 0.85abc	1.63 ± 0.81ab
T_{11} (1.5 + 0.2)	86.67 ± 5.77abide	15.00 ± 0.66abc	1.30 ± 0.44ab
T_{12} (1.5 + 0.3)	93.33 ± 5.77code	14.90 ± 0.70abc	1.47 ± 0.38ab
T_{13} (2.0 + 0.05)	96.66 ± 5.78de	13.97 ± 0.55ab	3.33 ± 0.38de
T_{14} (2.0 + 0.1)	100.00 ± 0.00e	13.27 ± 0.35a	4.77 ± 0.72f
T_{15} (2.0 + 0.2)	96.67 ± 5.77de	13.17 ± 0.55a	4.57 ± 0.78f
T_{16} (2.0 + 0.3)	86.67 ± 5.77abide	14.20 ± 0.40ab	4.07 ± 0.38ef
SE (m)	**2.12**	**0.55**	**0.18**

For each column, different superscript (Small alphabet) letters indicate significantly different at p≤0.05, as measured by Tukey's test between treatments.

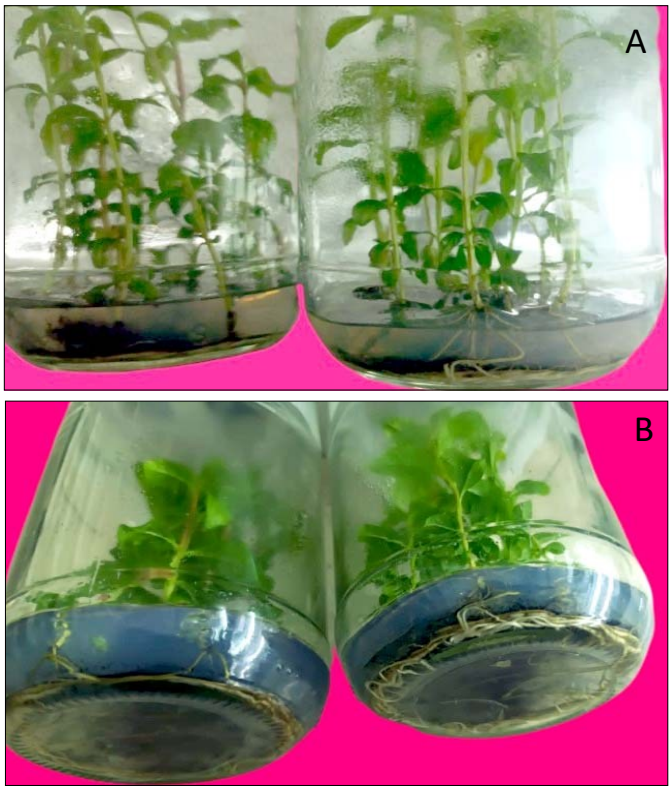

Fig. 2. *In vitro* root induction in pomegranate variety "Bhagwa" after 2 weeks of culture: (**A**) Control and (**B**) T_{14}-2.0 mg/L IAA and 0.1 mg/L SNP root developed in pomegranate plantlets.

44, 45). In recent times, SNP has also been used to improve root induction in various plant species, such as *Cucumis sativus,* cherry rootstocks, antirrhinum and *Canscora diffusa* (31, 46-48). SNPs act as signals in the auxin-induced signaling cascade that promotes adventitious root growth (49). Previous research also reported an increase in the number of roots when SNP was added to a medium supplemented with IAA (31). The high concentrations of auxin are necessary for rapid root initiation, which is only required during the initial phase of root development. Similar findings were observed in tomatoes when IAA was combined with different SNP combinations (50). SNPs play a crucial role in lateral root development. Cytokinin-induced NO production in plant cell cultures suggests the involvement of NO in cytokinin signal transduction (36). Therefore, the addition of the NO donor SNP promotes root formation. Previous research has also demonstrated that IAA is the primary auxin used by plants for adventitious rooting (51). Our *in vitro* rooting results are consistent with the previous findings, where IAA was used in combination with SNP for *in vitro* rooting of *Lycopersicon esculentum* Mill (50).

Assessment of genetic fidelity of in vitro grown micro-plants through ISSR markers

In the present study, a set of ten ISSR markers were employed to analyze micro-plants and the mother plant to evaluate the genetic fidelity of micropropagated pomegranate plantlets. Initially, DNA fingerprint analysis was conducted using 10 primers and 6 primers generated distinct and reproducible amplified fragments (Table 5). Marker analysis revealed that a significant proportion (88.8 %) of the amplicons derived from the *in vitro* cultured plants exhibited the same genetic makeup as their parental plants (Fig. 3a and b). The ISSR primers produced a range of 2 to 4 bands, with an average of 2.6 monomorphic bands per primer (Table 5). Among the 6 ISSR primers used, a single primer, UBC868, exhibited four bands, 2 of which were polymorphic, while the remaining primers resulted in 16 monomorphic bands, indicating genetic fidelity among the micro-propagated plants, while a little polymorphism (11.1 %) for the UBC868 primer was also observed. The 5 other primers exhibited a consistent mono-

Table 5. Primers, size range (bp) and amplified fragments generated through ISSR primers in micro-plantlets and mother plants of pomegranate.

Sl. No.	Primers	Total alleles	Polymorphic bands	Monomorphic bands	Band range (bp)
1.	UBC 831	4	0	4	250, 300, 380, 420
2.	UBC 873	3	0	3	200, 280, 340
3.	UBC868	4	2	2	200, 300, 400, 450
4.	IS7	2	0	2	100, 180
5.	IS15	3	0	3	150, 200, 300
6.	IS25	2	0	2	150, 290
Total		18.003.0	2.00	16.00	
Avg.		3	0.33	2.66	

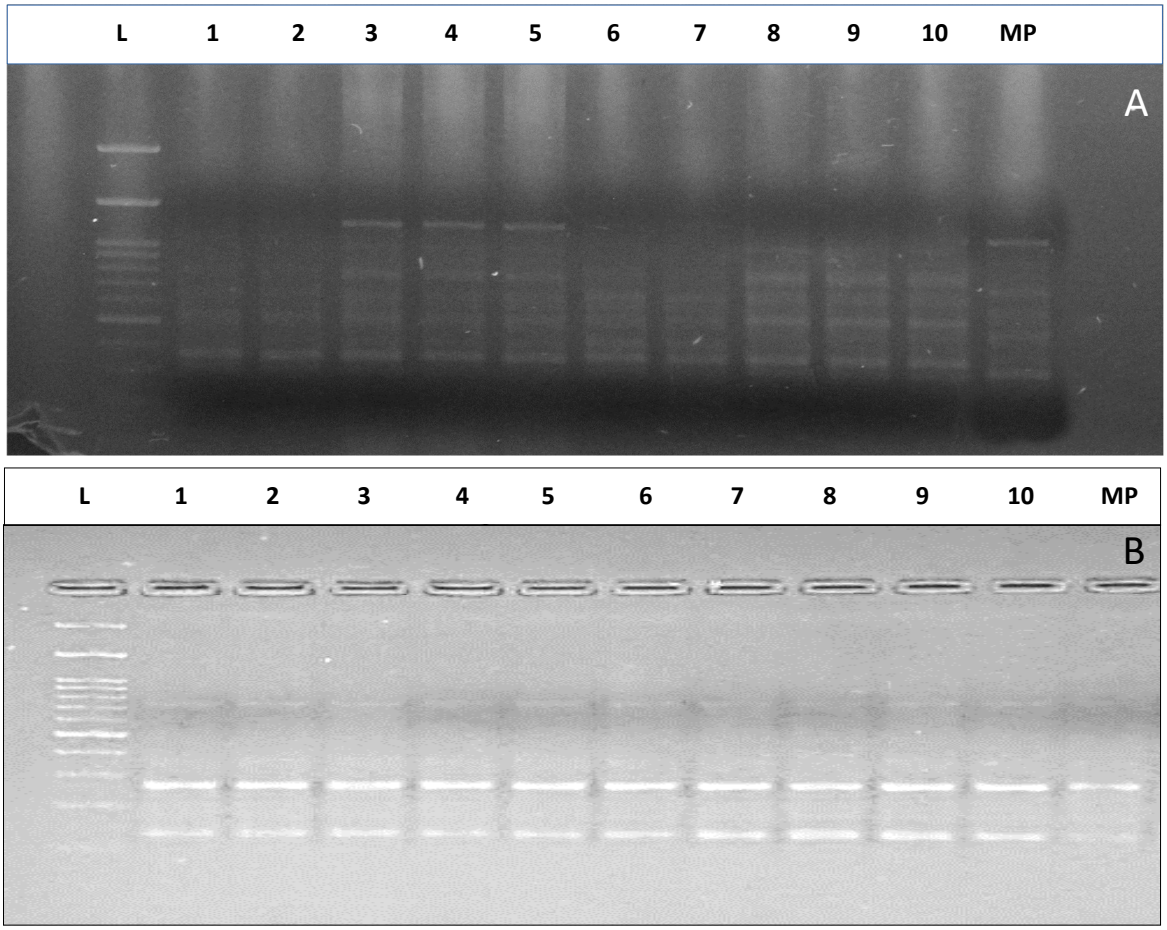

Fig. 3. Polymerase chain reaction (PCR) amplification products obtained through ISSR primer (**A**) (UBC 868) and (**B**) (IS 25) in mother plant and micro propagated plants of pomegranate; **L**: 100 bp Ladder: Lane 1-10 SC1- SC10 (SC: Sub culture); **MP**: Donor Mother Plant (DMC).

morphic pattern.

When aiming for complete genetic uniformity in regenerated plants, somaclonal variation can occur as an undesirable outcome of tissue culture. However, it can also serve as a novel source of genetic variability for breeding purposes (20). It is crucial to identify and check the extent of genetic variation in both scenarios. For checking somaclonal variation in micropropagated plants, molecular markers are an effective technique for achieving this goal (52). ISSR (Inter-Simple Sequence Repeat) markers are particularly convenient to use compared to other markers like RFLP, SSR and AFLP because they do not require prior sequence information to generate DNA amplification products (53). Polymorphism in amplification products, represented by 2 alleles, can occur due to alterations that affect the primer binding site sequence (such as point mutations) or that alter the size or prevent successful primer binding to the target DNA (54). The low genetic polymorphism observed in this study could be attributed to a limited amount of genetic variation, which can be induced by factors such as prolonged culture times, specific combinations of plant growth hormones or stress caused by added biochemicals known to induce somaclonal variation in tissue-cultured plants. These findings are closely related to studies on *in vitro* propagated *Guizotia abyssinica* Cass, *Miscanthus* X *giganteus* and *Dendrobium chrysotoxum,* which also reported genetic similarity among the micropropagated plants (54-56). The genetic fidelity of micropropagated jojoba plants compared to their mother plants using RAPD and ISSR markers demonstrated a 100 % simi-

larity. Their study highlighted that axillary bud multiplication is a reliable method for producing true-to-type plants (57). Similarly, in another study, researchers used nine ISSR primers, generating 56 clear, distinct and reproducible amplicons. Cluster analysis revealed 100 % genetic similarity between the mother plant and its derived regenerants within the same cluster (58). In a similar study, 100 % genetic fidelity was also found between *in vitro* propagated Gerbera plants and the mother plant using RAPD and ISSR markers (59).

Conclusion

Most plants grown *in vitro* exhibited genetic uniformity with their maternal plants, although some somaclonal variation was observed. This is the first report to use SNP in Punica for *in vitro* culture. This study recommends using *in vitro* culture procedures to check clonal fidelity before commercial planting to ensure true-to-type plants. ISSR markers were employed to identify genetically similar plants grown *in vitro* using SNP with other PGRs. An efficient protocol for micropropagation of pomegranate cv. 'Bhagwa' using nodal segments as explants was established, with ISSR markers revealing that 88.89 % of the micro-propagated shoots were genetically uniform, showing only 11.11 % somaclonal variation. These findings can help reduce time and costs for researchers and stakeholders in the plant tissue culture industry and can be applied to other fruit crops for commercial production.

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Authors' contributions

MK and MKY designed the experiment. DP and CC carried out the experiment. DP, AP and VR did tissue culture work. DP, CC and VP performed molecular work. VP and AP performed statistical analysis. CC and VR wrote and reviewed the manuscript. All authors read and approved the final version.

Compliance with ethical standards

Conflict of interest: The author (s) declares no conflict of interest.

Ethical issues: None.

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