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RESEARCH ARTICLES

Effect of various chemical solutions on micropropagation efficiency, antioxidant activities and clonal fidelity analysis of in vitro developed plantlets in pomegranate (*Punica granatum* L.) using SSR markers

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Abstract

This study aimed to develop an effective in vitro protocol for micropropagation of pomegranate (*Punica granatum* L.) var. Bhagwa using nodal explants. To induce shoot growth and cultivating the explants, Murashige and Skoog's media were supplemented with benzyl amino purine (BAP), sodium nitroprusside (SNP), an approach demonstrated and experimented for the first time on Pomegranate and kinetin (Kn) in various concentrations and combinations. In the MS medium fortified with 1.5 mg/l BAP, 1.5 mg/l SNP, and 1.5 mg/l Kn, maximum shoot length, shoot per explants, and regeneration % were noted. For root induction, uniform micro shoots were separated and then placed in the rooting media (Murashige and Skoog medium at half strength), supplemented with different ratios of NAA and SNP with 2 mg/L NAA and 0.3 mg/L SNP yielding the highest culture rooting, number of roots, and root length. The described methodology permits the development of multiple pomegranate plants that have been micro propagated. Superoxide dismutase, catalase, and guaiacol peroxidase activities revealed the antioxidant defence mechanism of micro propagated pomegranate micro shoots, which increased shoot development and the number of shoots per explant. Using simple sequence repeat (SSR) markers, the mother plants and proliferating plantlets were checked for genetic integrity; the results showed that all of the plants were the same. The results of this study could be used commercially in the future to produce high-quality pomegranate planting material.

Keywords Antioxidant · Biochemical analysis genetic fidelity · Micropropagation · Pomegranate · SSR markers

Abbreviations

BAP/BA	Banzyl amino purine/ Benzyl adenine
NAA	Napthaline acetic acid
SNP	Sodium Nitro Prusside
MS	Murashige and Skoog
Kn	Kinetin
SOD	Superoxide dismutase
CAT	Catalase
GPO	Guaiacol peroxidise
CTAB	Cetyl Triethyl Ammonium Bromide
CRD	Completely randomized design
ANOVA	Analysis of variance

Introduction

Pomegranate (*Punica granatum* L., *Punicaceae*) is an economically important fruit crop grown in tropical and subtropical regions of the world (Jurenka 2008). India is the largest producer of pomegranates in the world, about in 2.82 lakh hectares, producing 32.16 lakh MT (NHB 2022). In India, Maharashtra top-producing state for pomegranate followed by Karnataka, Andhra Pradesh, Gujarat, Tamil Nadu, and Rajasthan states are commercial producers of pomegranate fruits.

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Pomegranate Bhagwa is a popular variety and it is huge, spherical fruit with bright crimson arils and delicate seeds. It is currently farmed in India, Iran, Turkey and United States. It has abundance in antioxidants, vitamins, and minerals makes it a fruit that is both nourishing and healthy (Singh et al. 2018; Yassin et al. 2021). It is primarily used for fresh consumption and RTS (Ready to Serve) Beverages, and its demand has been steadily increasing due to its high commercial value (Shelar et al. 2017.

). In the view of increasing demand, there is need of commercial production of high-quality planting material of pomegranate. Conventional propagation methods, such as seed germination and cutting, are often limited by low multiplication rates, seasonality, and the risk of endemic diseases (Kumar et al. 2018). Hardwood and softwood cuttings are the traditional methods for propagating pomegranates, although they do not guarantee disease-free and healthy plants (Anon 1982; Da Silva et al. 2013b). Therefore, in vitro micropropagation offers a promising alternative for rapid multiplication of healthy, disease-free plants throughout the year (Kaur and Kumar 2020).

Now-a-days, micropropagation techniques are used to for rapid production of disease-free plants desirable traits (Zayova et al. 2018). When different potential plant growth regulators (PGRs) are added to the culture medium in varying doses and combinations, different explants and genotypes may have a greater effect on the regeneration of plantlets. It is crucial to enhance regeneration protocols to address problems with clonal propagation and gene transformation techniques in order to satisfy the rising demand for pomegranates (Arun et al. 2017). However, the appearance of soma clonal variation is a possible disadvantage when propagating a species of elite plants. The clonal fidelity, or genetic homogeneity, of the plantlets created during micropropagation must be examined to maintain the intended elite genotypes (Maurya et al. 2021). Using molecular markers, which are used to check the genetic differences between mother plants and micro-plantlets, it is possible to examine the genetic fidelity of plants that have been grown in lab conditions (Jin et al. 2012; Singh et al. 2013b).

Efficient in vitro micropropagation of pomegranate requires the development of optimized protocols for shoot induction, multiplication, and rooting of explants. Various factors, such as media composition, plant growth regulators, and explant type, can affect the success rate of micropropagation and the quality of the resulting plantlets. Furthermore, maintaining clonal fidelity, genetic uniformity, and high antioxidant activity of micro propagated plants is crucial for ensuring their suitability for commercial applications (Debnath 2017, Yassin 2021). The interaction of ROS with several plant growth regulators confirms the impact of ROS on plant growth and development which can also promote a range of physiological disorders (Joyce et al. 2003). Furthermore, ROS have been identified as a secondary signal for various plant hormone responses. As a result, plants have evolved antioxidant systems to defend themselves against all these threats as scavenging reactive oxygen species (ROS) during plant metabolism (Niki 2011; Tubic et al. 2016). Numerous enzymes and low-molecular-weight metabolites provide antioxidant protection at the cellular and organism levels (ascorbic acid, uric acid, glutathione, tocopherols, and several others). Based on recent studies, Sodium nitroprusside (SNP) also known as Nitric oxide (NO) and Napthaline acetic acid (NAA) used to enhance in vitro plant regeneration (Lau et al. 2021).

This study aimed to develop an efficient in vitro protocol for micropropagation of pomegranate using nodal explants of the Bhagwa variety. The protocol was optimized for shoot induction and multiplication using a combination of plant growth regulators, and rooting was induced using different ratios of NAA and SNP. The antioxidant activity of micro propagated plants was assessed through the activities of antioxidant enzymes, and the genetic fidelity was confirmed using SSR markers. The findings of this study hold promise for the development of an efficient in vitro protocol for micropropagation of pomegranate with increased antioxidant activity and genetic uniformity. The optimized protocol may contribute to the commercial production of high-quality planting material for pomegranate crops.

Materials and methods

The study was carried out over a period of three years, starting from August 2019 and concluding in June 2022. The research was conducted at the plant tissue culture laboratory of the College of Horticulture, S. V. P. University of Agriculture & Technology, Meerut (U.P.), India 250 110.

Collection of explants and surface sterilization

The stem cuttings were collected from the healthy donor plants and further in vitro culture was done from the nodes of these cuttings. Nodal explants were exposed to Teepol (0.1%) for 10 min, Bavistin (0.1%) for 20 min, and 70% ethyl alcohol for a minute, and then washed 3–4 times with clean, running tap water. After pre-surface sterilization, explants were excised to around 4–5 mm in size and further transferred to the laminar air flow for post-sterilization. Before being placed on the culture medium, the explants were thoroughly washed three to four times with double-distilled water and sterilized with a 0.1% $HgCl_2$ solution for 1–2 min under a laminar airflow.

In vitro establishment of shoot formation and multiplication

Nodal explants that had been surface-sterilized were put on MS media containing Benzyl Amino Purine (BAP), Sodium Nitroprusside (SNP), and Kinetin in various formulations in to initiate shoot production in vitro. Healthy shoots with a length of 2.5 and 3.0 cm were chosen for further multiplication and placed on Murashige and Skoog (1962) basal media containing 27 treatment combinations of BAP, SNP, and Kn. All the cultures, namely T_1–T_{27}, were incubated at 25 ± 2^0C in a growth chamber with a 16–8-hour photo/dark cycle for a period of four weeks. The development of shoot buds were observed and recorded in order to check the cultures were closely monitored, and the data was recorded after 4 weeks of incubation for further analysis.

In vitro root formation of micro-plantlets

Micro-shoots that had been in vitro multiplied were put into MS medium that included varying concentrations of NAA and an SNP combination to encourage the emergence of roots. Sixteen different concentration combinations of NAA with SNP were used on the Murashige and Skoog (1962) medium with control, and the media were named T1–T16. All cultures were kept at 25 ± 2^0C in a growth chamber with a photo/dark cycle of 16/8 hours. Observations of two-week-old cultures of were recorded for root formation.

Antioxidant assays

Dry plant samples (0.3 g) from four-week-old micro-propagated plantlets grown in tissue culture with varied PGR concentrations were used for antioxidant analysis. The plant samples were subjected to examination of superoxide dismutase (SOD), catalase (CAT), and guaiacol peroxidase (GPO) antioxidant enzymes, utilizing the methodology described by Hristozkova et al. (2017). Total SOD (EC 1.15.1.1) activity was determined using the technique developed by Giannopolitis and Ries (1977). Complete CAT (EC 1.11.1.6) activity was observed through the use of Beers and Sizer's technique (1952). Total GPO (EC 1.11.1.7) activity was measured in accordance with the method described by Urbanek et al. (1991).

DNA isolation and selection of SSR primers

Genomic DNA from mother plants and microplants was extracted using standard protocols (Doyle and Doyle 1990). 0.8% agarose gel was used for electrophoresis of isolated DNA samples and further purity of the DNA was tested using a Smart SpecTM Plus spectro-photometer from Bio-Rad. Amplicons were run through a PCR (polymerase chain reaction) in accordance with the protocol using an electrophoresis apparatus with an 80-volt electric current. The sequences of the seven SSR primers that were employed in the current study were borrowed and synthesized from the publication of Hasnaoui et al. (2010).

Clonal fidelity analysis

To assess the clonal fidelity of mother plants and in vitro plantlets, seven SSR primers were used. Leaf tissue from the mother plant and in vitro-regenerated plants was used to create separate DNA preparations. Band intensities on an agarose gel containing 0.8% were used to measure the quantity and quality of DNA samples. To compute and assess the clonal fidelity, only distinct and scorable bands were used.

Statistical analysis and experimental planning

Three replications of the experiment were carried out using a completely randomized experimental design (CRD). The data were statistically analyzed using one-way analysis of variance (ANOVA), and Duncan's multiple test range ($P \leq 0.05$) was then applied. ($P \leq 0.05$) was used to determine significance in comparison to either the control or the PGRs or chemical treatments. SSR markers produced bands that were manually scored on the gel based on the presence (1) or absence (0) of repeatable and scorable bands.

Results and discussion

The effect of Benzyl Amino Purine (BAP), Sodium Nitroprusside (SNP), and Kinetin (KN) supplemented in MS medium in different combinations was studied in pomegranate cv. Bhagwa. The parameters of the present study included time taken for shoot formation (days), shoot regeneration (percent), number of shoots, and length of shoot recorded after 35 days. Twenty-seven concentration combinations of plant growth regulators named T_1–T_{27} were used in MS media. Each culture contained a single nodal explants inoculation to encourage the growth of buds and shoots. The results revealed that when media were supplemented with different concentration combinations of BAP, SNP, and Kn, treatment T_{27} (1.5 mg/l BAP, 1.5 mg/l SNP, and 1.5 mg/l Kn) showed the highest shoot regeneration percentage (86.67 ± 5.77; (Table 1; Fig. 1). It is also observed that the maximum shoot regeneration percentage might be due to the application of cytokinin when used along with an auxin, as per Hesami et al. (2019). Optimization of balance with respect to dose and concentration of SNP is important for maximum shoot regeneration percentage, as it is well

Table 1 Effect of SNP, BAP and Kinetin on in vitro shoot induction, shoot regeneration, number of shoots and shoot length of explants of Pomegranate cultivar Bhagwa

Treatment (SNP+BAP+KN mg/l)	Time taken for shoot induction (Days)	Shoot regeneration (percent)	Number of shoots	Length of shoot recorded after 35 days (cm)
Control	29.95 ± 1.4^{bcd}	36.67 ± 7.79^{a}	0.685 ± 0.125^{a}	0.62 ± 0.18^{a}
T1(0.5 + 0.5 + 0.5)	24.03 ± 1.89^{abc}	80.00 ± 0.00^{bcd}	1.73 ± 0.47^{abde}	2.67 ± 0.51^{bc}
T2(0.5 + 0.5 + 1.0)	30.00 ± 1.55^{cd}	63.33 ± 5.77^{abcs}	1.17 ± 0.31^{abc}	1.67 ± 0.15^{ab}
T3(0.5 + 0.5 + 1.5)	28.73 ± 3.06^{abcs}	53.33 ± 5.77^{abc}	0.73 ± 0.12^{a}	1.37 ± 0.51^{ab}
T4(0.5 + 1.0 + 0.5)	29.53 ± 3.01^{abcs}	63.33 ± 5.77^{abcs}	0.93 ± 0.38^{a}	0.90 ± 0.20^{a}
T5(0.5 + 1.0 + 1.0)	29.87 ± 1.30^{bcd}	56.67 ± 11.55^{abcs}	0.57 ± 0.32^{a}	0.93 ± 0.15^{a}
T6(0.5 + 1.0 + 1.5)	30.57 ± 2.12^{d}	53.33 ± 5.77^{abc}	1.83 ± 0.31^{abde}	1.70 ± 0.40^{ab}
T7(0.5 + 1.5 + 0.5)	29.77 ± 1.63^{bcd}	50.00 ± 0.00^{ab}	1.20 ± 0.30^{abcs}	1.07 ± 0.15^{ab}
T8(0.5 + 1.5 + 1.0)	28.20 ± 1.87^{abcs}	63.33 ± 5.77^{abcs}	2.43 ± 0.40^{cdefg}	1.33 ± 0.57^{ab}
T9(0.5 + 1.5 + 1.5)	28.60 ± 1.73^{abcs}	76.67 ± 11.55^{abcs}	2.83 ± 0.25^{efgh}	1.83 ± 0.32^{ab}
T10(1.0 + 0.5 + 0.5)	30.07 ± 0.81^{cd}	63.33 ± 5.77^{abcs}	0.97 ± 0.21^{a}	1.03 ± 0.25^{a}
T11(1.0 + 0.5 + 1.0)	30.20 ± 2.26^{cd}	50.00 ± 0.00^{ab}	0.87 ± 0.21^{a}	1.67 ± 0.60^{ab}
T12(1.0 + 0.5 + 1.5)	30.83 ± 2.12^{d}	56.67 ± 15.28^{abcs}	2.40 ± 0.56^{cdefg}	1.63 ± 0.35^{ab}
T13(1.0 + 1.0 + 0.5)	29.2 ± 1.39^{abcs}	50.00 ± 10.00^{ab}	1.030 ± 0.25^{ab}	1.13 ± 0.21^{ab}
T14(1.0 + 1.0 + 1.0)	29.07 ± 3.44^{abcs}	50.00 ± 20.00^{ab}	3.00 ± 0.10^{efgh}	1.37 ± 0.60^{ab}
T15(1.0 + 1.0 + 1.5)	26.2 ± 1.40^{abcs}	76.67 ± 11.55^{abcs}	2.93 ± 0.4^{efgh}	1.83 ± 0.50^{ab}
T16(1.0 + 1.5 + 0.5)	30.63 ± 2.38^{d}	46.67 ± 20.82^{a}	2.47 ± 0.45^{deg}	1.90 ± 0.26^{ab}
T17(1.0 + 1.5 + 1.0)	29.77 ± 2.10^{bcd}	76.67 ± 5.77^{abcs}	2.80 ± 0.20^{efgh}	1.60 ± 0.50^{ab}
T18(1.0 + 1.5 + 1.5)	23.77 ± 1.32^{ab}	86.67 ± 5.77^{d}	3.67 ± 0.45^{ghi}	3.70 ± 0.95^{cd}
T19(1.5 + 0.5 + 0.5)	29.57 ± 1.32^{abcs}	53.33 ± 15.28^{abc}	0.90 ± 0.20^{a}	1.37 ± 0.49^{ab}
T20(1.5 + 0.5 + 1.0)	30.4 ± 2.11^{d}	46.67 ± 15.28^{a}	2.30 ± 0.20^{bcdef}	1.87 ± 1.25^{ab}
T21(1.5 + 0.5 + 1.5)	27.53 ± 3.27^{abcs}	83.33 ± 5.77^{cd}	3.23 ± 0.25^{fgh}	1.87 ± 0.49^{ab}
T22(1.5 + 1.0 + 0.5)	30.9 ± 1.90^{d}	50.00 ± 10.00^{ab}	1.43 ± 0.55^{abcs}	1.37 ± 0.35^{ab}
T23(1.5 + 1.0 + 1.0)	27.3 ± 1.15^{abcs}	80.00 ± 10.00^{bcd}	3.03 ± 1.08^{efgh}	1.70 ± 0.46^{ab}
T24(1.5 + 1.0 + 1.5)	24.10 ± 2.62^{abc}	83.33 ± 5.77^{cd}	4.03 ± 0.21^{hi}	3.63 ± 0.51^{cd}
T25(1.5 + 1.5 + 0.5)	27.57 ± 0.67^{abcs}	80.00 ± 10.00^{bcd}	2.87 ± 0.32^{efgh}	1.80 ± 0.61^{ab}
T26(1.5 + 1.5 + 1.0)	25.33 ± 0.80^{abcs}	83.33 ± 5.77^{cd}	3.93 ± 1.11^{hi}	3.57 ± 0.50^{cd}
T27(1.5 + 1.5 + 1.5)	23.53 ± 0.93^{a}	86.67 ± 5.77^{d}	4.53 ± 0.55^{i}	4.1 ± 0.56^{d}
SE (m)	**0.32**	**1.83**	**0.13**	**0.11**

Significant changes between treatments are indicated by letters on the superscript ($P < 0.05$)

Fig. 1 In Vitro shoot induction in pomegranate cv. "Bhagwa" after 4-weeks of culture. (**A**) Control. (**B**) T-20–0.5 mg/l BAP (1.5 mg/l SNP and 1.0 mg/l Kn). (**C**) T27-1.5 mg/l BAP, 1.5 mg/l SNP and 1.5 mg/l Kn

documented that SNP reduces the regeneration capacity at high doses when applied in medium. This is in accordance with study conducted by Xu et al. (2009). However, the type and concentration of PGRs (SNP, BAP, and KN) used in the study significantly influenced shoot regeneration as per the report of Naik et al. (2000) in pomegranate. In the past, BAP and Kinetin applications were quite efficient at regenerating several shoots in pomegranates (Singh et al. 2013a) Our findings are consistent with those of (Naik et al. 1999; Murkute et al. 2004; Naik and Chand 2011; Patil et al. 2011; Silva et al. 2013b) in pomegranates. A crucial phase in the development of a plant's regeneration system is the establishment of shoot and root regeneration in media. SNP (Sodium Nitroprusside), a Nitric Oxide (NO) donor, is crucial for in vitro propagation and is particularly successful at promoting the shooth growth which in align with Karthik et al. (2019) to our current study. Pomegranate micropropagation is the best approach for producing high-quality, disease-free plant material, but no research has been done on the effect of PGRs added to the culture media on plant antioxidant capacity. The most critical criteria for successful pomegranate propagation are determining the optimum type and concentration of plant growth regulator, which has a major effect on the percentage of shoot creation and the number of shoots per explants. There have been numerous studies done to determine the ideal formulations of plant growth regulators for pomegranate shoot and root differentiation as well as plant regeneration (Naik et al. 2000; Kaji et al. 2013; Singh et al. 2013a; Bachake et al. 2019; Abadi et al. 2020).

Many factors and substances, such as the type of auxin utilized, have a significant impact on the rooting of proliferating micro-shoots. The maximum (100.00 ± 00) of rooting regeneration was observed in treatments T_{10}, supplemented with (NAA 1.5 mg/l and SNP 0.1 mg/l), T_{11} (NAA 1.5 mg/l and SNP 0.2 mg/l), and T_{12} (NAA 1.5 mg/l and SNP 0.3 mg/l), while the minimum (63.33 ± 50) was observed in treatment T_1 supplemented with NAA (0.5 mg/l) and SNP (0.05 mg/l). (Table 2; Fig. 2). In the present study, maximum root regeneration percentage was observed in optimized media containing combinations of PGRs, which might be due to the significant variations among the treatments or also be due to the correlation between auxin level and nodal segment position on the shoot as reported by Al-Wasel (1998) in pomegranate. The minimum root regeneration percentage recorded may be the cause of roots being induced on shoots cultured on media with low hormonal levels,. Earlier research in pomegranates (El-Agamy et al. 2009; Deepika and Kanwar 2010) found similar results. When media were formulated by varying concentrations of IAA and SNP in combinations, the maximum (100.00 ± 00) percent regeneration of rooting was recorded in treatment T_{29}, which was fortified by IAA (2.0 mg/l) and SNP (0.1 mg/l), while the minimum rooting percent (63.33 ± 05) was observed in treatment T_{19}, which was fortified with IAA (0.5 mg/l) and SNP (0.2 mg/l).

Table 2 Effects of NAA and SNP on *in vitro* root initiation, root development and number of roots per explants of pomegranate cultivar Bhagwa

Treatment (NAA + SNP mg/l)	Root development (percent)	Time taken for root initiation (Days)	Number of roots/explants
Control	70.00 ± 17.32^{a}	22.8 ± 2.52^{f}	1.43 ± 0.15a
T1(0.5 + 0.05)	83.33 ± 5.77^{ab}	17.37 ± 1.53^{e}	1.93 ± 0.15^{abc}
T2(0.5 + 0.1)	80.00 ± 10.00^{ab}	17.03 ± 1.63^{e}	2.07 ± 0.32^{abc}
T3(0.5 + 0.2)	93.33 ± 5.77^{ab}	16.23 ± 0.93^{de}	2.37 ± 0.42^{abc}
T4(0.5 + 0.3)	90.00 ± 10.00^{ab}	16.43 ± 0.72^{de}	2.63 ± 0.29^{bc}
T5(1.0 + 0.05)	90.00 ± 17.32^{ab}	15.93 ± 0.71^{code}	3.00 ± 0.10^{c}
T6(1.0 + 0.1)	90.00 ± 10.00^{ab}	15.50 ± 0.30^{bode}	2.43 ± 0.42^{abc}
T7(1.0 + 0.2)	96.67 ± 5.77^{ab}	15.97 ± 0.67^{code}	2.13 ± 0.32^{abc}
T8(1.0 + 0.3)	96.67 ± 5.77^{ab}	16.23 ± 0.99^{de}	2.03 ± 0.40^{abc}
T9(1.5 + 0.05)	90.00 ± 10.00^{ab}	15.27 ± 0.80^{bode}	1.83 ± 0.25^{ab}
T10(1.5 + 0.1)	100.00 ± 0.00^{b}	14.80 ± 0.70^{abide}	1.93 ± 0.15^{abc}
T11(1.5 + 0.2)	100.00 ± 0.00^{b}	14.60 ± 0.56^{abide}	1.73 ± 0.25^{ab}
T12(1.5 + 0.3)	100.00 ± 0.00^{b}	15.30 ± 0.61^{bode}	1.73 ± 0.32^{ab}
T13(2.0 + 0.05)	86.67 ± 5.77^{ab}	13.60 ± 0.46^{abcs}	4.13 ± 0.35^{d}
T14(2.0 + 0.1)	86.67 ± 11.55^{ab}	12.97 ± 0.57^{ab}	5.27 ± 0.55^{e}
T15(2.0 + 0.2)	90.00 ± 17.32^{ab}	12.10 ± 0.46^{a}	6.20 ± 0.66^{f}
T16(2.0 + 0.3)	93.33 ± 5.77^{ab}	13.07 ± 0.38^{abc}	6.40 ± 0.72^{f}
SE (m)	**1.56**	**0.35**	**0.22**

Significant changes between treatments are indicated by letters on the superscript ($P < 0.05$)

Fig. 2 In vitro root induction in pomegranate variety "Bhagwa" after 2 weeks of culture: (**A**) Control and (**B**) T10-1.5mg/l NAA and 0.1mg/l SNP (**B**) root developed in pomegranate cv Bhagwa plantlets

Antioxidant activity on in vitro propagated pomegranate

The enzyme activities and antioxidant capacity of extracts generated from in vitro propagated pomegranate plant samples were modified in different ways as a result of the absence (control medium) or presence of different plant growth regulators added to the culture medium. Plantlets cultivated on MS medium without PGRs showed low activity of the enzymes SOD, CAT, and GPO. However, adding BAP, SNP, and Kn to the MS medium resulted in enhanced enzyme activity. Out of 27 treatments, six were optimized, besides the control, for assays of enzyme activity on the basis of shoot generation percentage in in vitro pomegranate plants. In treatment T_{27}, the enzyme activities of SOD, CAT, and GPO were higher when compared with other treatments (Fig. 3). It is well documented that the production of reactive oxygen species (ROS), which changes enzyme function, is one of the earliest such processes occurring at the cellular level. In plants, the production and detoxification of ROS occur in close synchrony, and a sophisticated antioxidant system tightly regulates the amounts of both. Plantlets that were grown in adverse conditions such as high humidity, warmth, and light exposure during in vitro culture resulted in aberrant morphology, anatomy, or physiology (Hazarika 2006; Cramer et al. 2011). Our findings were partially consistent with the previous one, which demonstrated that the presence of BAP, Kinetin, and SNP in MS media increased the enzyme activities of in vitro plantlets in various crops (Zayova et al. 2019; Cuce and Muslu 2022; Sichanova et al. 2022). Also, it was discovered that increasing SNP, BAP, and kinetin concentrations in MS medium led to an increase in enzyme activity, which denoted plant stress. Recent research on a variety of plant species, including strawberries (Tian et al. 2003), H. officinalis (Zayova et al. 2018), and Atropa acuminata (Dar et al. 2022), has shown that the ability to create new shoots and regenerate is improved when antioxidant enzyme activity is increased. Furthermore, according to Wojtania and Skrzypek (2014), the activity of enzymes like catalases and peroxidases, which catalyse the breakdown of H2O2 into water and oxygen in Pelargonium shoots, was favourably influenced by cytokinins like BAP.

Since it is widely known that CAT and peroxidases play a significant role in plant development and differentiation, it is possible that their high activity during in vitro propagation will contribute to the process of differentiation through the induction of shoots (Molassiotis et al. 2004). The interaction between ROS and plant growth regulators maintains the role that ROS plays in plant growth and development. They have also been connected to the second messenger in a number of plant hormone responses (Kwak et al. 2006). To take into account the variations in the activities of antioxidant enzymes during the in vitro culture period based on the type of PGR or its concentration in the culture, comparative research of the effects of various PGR combinations has been conducted. The combination of cytokinin BAP with SNP and Kn in the culture medium increased the activities of antioxidant enzymes such as SOD, CAT, and GPO. As a result, both shoot development and the number of shoots per explant increased (Guranna et al. 2018).

Evaluation of the clonal fidelity of micro-plants

It is well known that the source of the explants and the regeneration techniques affect the genetic fidelity during in vitro propagation (Goto et al. 1998). Nodal explants are one of the

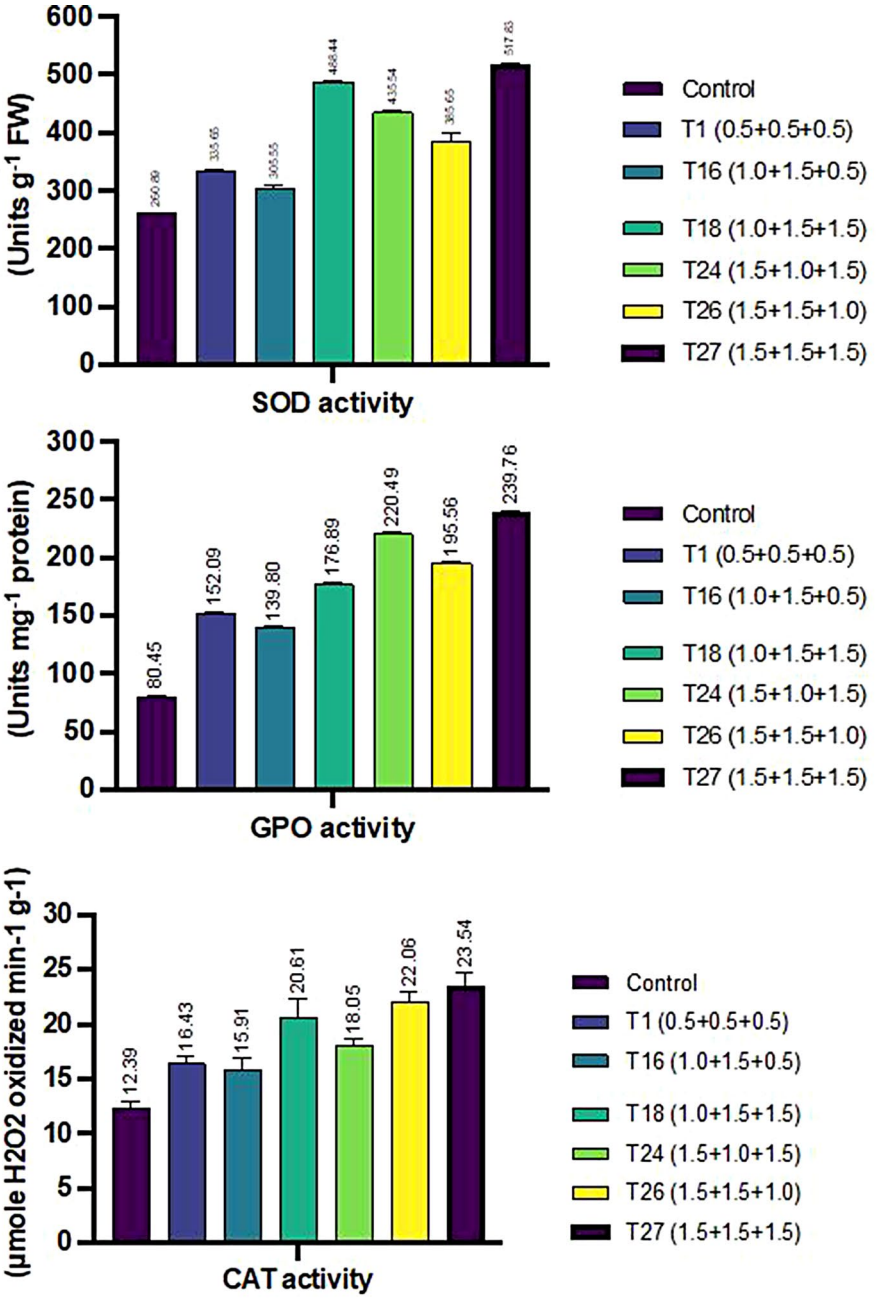

Fig. 3 The activity of antioxidant enzymes superoxide dismutase (SOD), guaiacol peroxidase (GPO) and catalase (CAT) in the extract from in vitro propagated pomegranate cv Bhagwa on the MS supplied with BAP, SNP and Kn at different concentrations

most popular in vitro propagation explants because of their ease of application and rapid rate of multiplication (Rani et al. 1995; Martins et al. 2004). Moreover, these plants have the least chance of genetic diversity. Martin et al. (2006) noted that although plants generated from these tissues are frequently genetically identical, variations in micro-propagated plants may be caused by plant growth hormones. Furthermore, as reported in several other crop species (De Laia et al. 2000; Devarumath et al. 2002), a well-organized meristem does not necessarily produce plants that are genetically similar to the mother plants.

To determine the genetic integrity of micro-plantlets of pomegranate plantlets, ten SSR markers were firstly screened, and seven SSR primers that showed the best amplification were finally used in eleven randomly selected plants as a separate subculture (SC). In the examined micro-plants, the SSR primers produced 1–2 bands, with an average of 1.57 bands per primer pair (Table 3 and Fig. 4). Two of the seven SSR primer pairs displayed a single band during the amplification procedure, while the other five showed double bands. A total of 11 bands (number of plants multiplied by the number of bands that could be scored by all primers) were obtained from in vitro-generated plantlets,

Table 3 Total number and size range of amplified fragments generated through SSR primers in micro propagated and mother plants of Pomegranate cv. Bhagwa

S. No.	Primers	Total alleles	Polymorphic Bands	Total number of amplified bands	Band size range
1.	Pom 039	2	0	22	100–300
2.	Pom 55	1	0	11	100–200
3.	Pom 46	1	0	11	100–200
4.	Pom 006	2	0	22	100–200
5.	Pom 010	2	0	22	100–200
6.	Pom 021	2	0	22	100–200
7.	Pom 014	1	0	11	100–200
Total		11	0	121	

including the mother plant. All 123 bands for all primers used in a single generation were visible. Micro-propagated plants' banding profiles were all monomorphic and resembled those of the mother plant. The outcomes in this study made it abundantly evident that the plantlets were true to type, or like their mother plants, in nature. SSR markers are important for fidelity studies because they are highly polymorphic and can differentiate between even closely related individuals, allowing for accurate identification and tracking of genetic variation. According to earlier reports, SSR markers produced more consistent outcomes. For example, Maurya et al. (2021) used SSR markers to achieve 100% stability in carnations, while Jin et al. (2014) used SSR markers to achieve 100% consistency in the apple rootstock *Malus hupehensis* var. *pinyiensis*. The SSRs-based study demonstrates unequivocally that the regenerated micro-plants exhibited monomorphic patterns and matched their mother micro-plants exactly (Fig. 4). As SSR markers are co-dominant in nature, two alleles were obtained at each location. Two alleles can sometimes be seen as a single band under various circumstances. Our findings demonstrated the genetic equality of the diverse micro-plant subcultures. As all the micropropagated plantlets shared a common genetic ancestor with their respective mother plants, this strongly implies that no somaclonal variation occurred during in vitro generation of plant.

Conclusion

The study successfully optimized the micropropagation protocol of the pomegranate cultivar "Bhagwa" by identifying the optimal combinations of BAP, SNP, and KN. The optimized protocol not only maintained clonal integrity but also resulted in higher antioxidant enzyme activities compared to other treatments. These findings suggest that the developed micro-plant regeneration procedure is suitable for large-scale clonal propagation of the "Bhagwa" cultivar. This optimized micropropagation protocol can serve as a basis for the production of large-scale, high-quality pomegranate plants for commercial cultivation.

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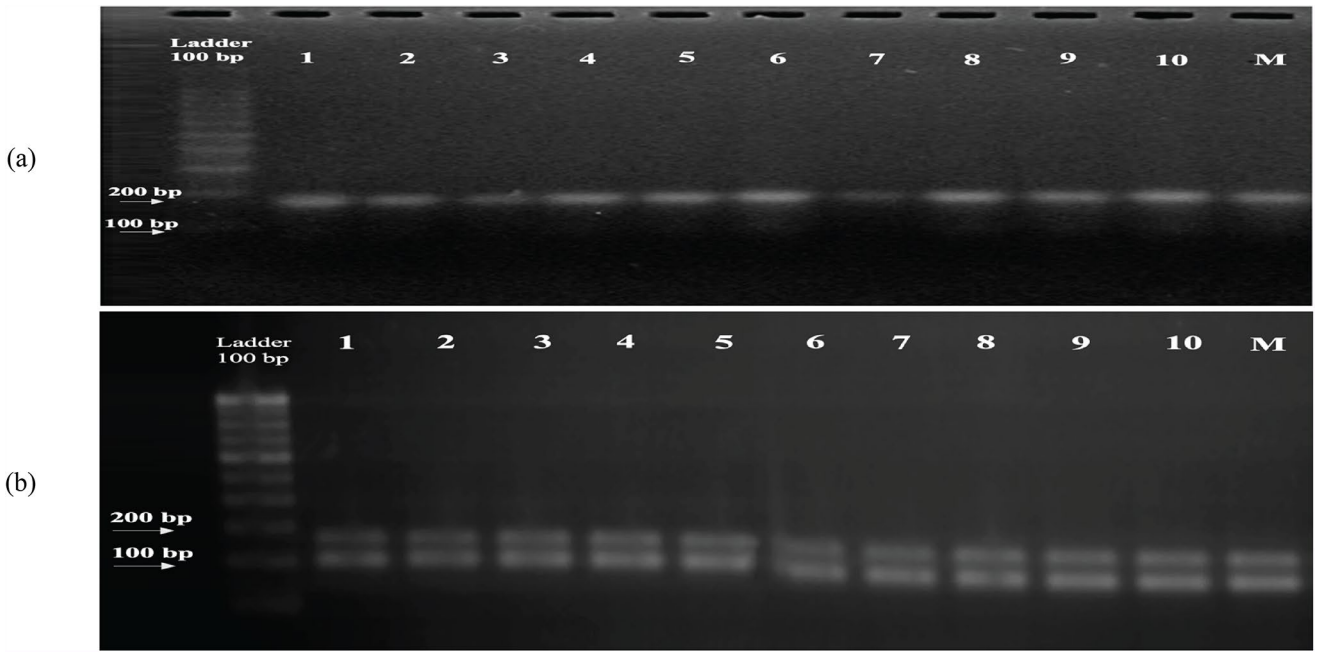

Fig. 4 DNA banding pattern generated through SSR primers (**a**) Pom 46 and (**b**) Pom 006 in mother plant and micro propagated plantlets of Pomegranate cv Bhagwa

Author contributions All the authors contributed equally in the present research manuscript.

Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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