J Plant Mol Breed (2021) 9(2): 43 - 51
DOI: 10.22058/JPMB.2022.561653.1262

RESEARCH ARTICLE

Effect of Sodium Nitroprusside and Some Plant Growth Regulators on Shoot Regeneration and Plantlet Development in *Lycopersicon esculentum* Mill.

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ABSTRACT: Improving plant regeneration skills in tissue culture studies is critical not only for the efficient genetic transformation of commercial crops but also for scientific reports. SNP (Sodium nitroprusside) as a Nitric oxide (NO) donor, plays an important role in the growth and development of plants. In this study, regeneration and plantlet development of *Lycopersicon esculentum* Mill. was improved using optimized concentrations of plant growth regulators supplemented with sodium nitroprusside. According to the results, among 12 different combinations of plant growth regulators, the MS medium complemented with 2 mg L^{-1} BAP and 0.2 mg L-1 IAA had a maximum percentage of regeneration (84%). The highest stem length (4.6 cm) and leave number (7) were achieved on MS medium supplemented with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} IAA. Adding of 10 µM sodium nitroprusside to the regeneration medium improved shoot regeneration efficiency (93%) and the number of shoots per explants (7.75). Furthermore, the maximum shoot growth mean, including stem length (11.8) and leaf number (11.2) were achieved on MS medium containing BAP (0.5 mg L^{-1}), IAA (0.2 mg L^{-1}), and 10 µM sodium nitroprusside. It was found that fewer adventitious roots and higher lateral roots were significantly developed in the medium containing IAA and SNP. Our findings indicated that adding SNP to the regeneration medium of *L. esculentum* Mill. improved shoot regeneration and plant development. This may overcome the problems in proliferation of the tomato plant.

KEYWORDS: Sodium nitroprusside, Shoot regeneration, Tomato, PGRs.

INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is a large, fleshy vegetable crop in the Solanaceae family. It has an extremely low sugar content, far less than other edible fruits (1). Tomato fruits provide nutrition to a majority of the world's population. Therefore, tomato is widely cultivated as a fresh vegetable and concentrated processed products for consumption (2). Tomato is high in vitamin A, vitamin C, fibers, and antioxidants such as flavonoids, lutein, lycopene, zeaxanthin, and β-carotenes (3). Carotenoid compounds such as lycopene protected the human cells and organs against harmful radicals, thus lowering cancer risk (4). Tomato production is affected by numerous abiotic and biotic stresses such as drought, high temperatures, salt stress and also pathogens. To solve the mentioned problems, tissue culture techniques can act as a fundamental instrument that can increase crop production through the rapid availability of desired plant materials (1). Further, tomato is the main vegetable crop for genetic engineering purposes and is regarded as a model for introducing agronomically essential genes into dicotyledonous crop plants (5). Successful genetic manipulation of plants is dependent mainly on the

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Received: 7 September 2022/ Revised: 26 November 2022
Accepted: 10 December 2022

accessibility of a reliable regeneration system. Moreover, propagation of heterosis varieties through tissue culture can be done using *in vitro* micropropagation that involves the selection of appropriate explants and the condition of suitable medium requirements (6, 7).

Auxins and cytokinins are key growth regulators for plant cellular differentiation or dedifferentiation. The combination of BAP with IAA or IBA has been used to be effective inducers of callus production and plant regeneration in tomato (8).

Nitric oxide (NO) is a widely distributed bioactive molecule that is required for a variety of plant growth pathways, such as flowering, fruit ripening, germination and senescence (9). Treatment of plants with NO can boost their resistance to different stresses, including dryness, salinity, temperature, UV radiation and heavy metals (10). As the main source of NO, sodium nitroprusside (SNP) is a phytohormone inducing plant growth and development, such as morphogenesis, seed dormancy reduction, lateral root growth, germination, shoot regeneration, root formation and senescence (11, 12). Due to the low cost and high duration of NO production, SNP is usually considered a preferred NO donor (13). SNP supplementation is documented to enhance callogenesis and multiple shoot regeneration (14). Adventitious root formation induced by exogenous SNP has been previously confirmed in the tomato plant (15). Interaction between plant growth regulators and SNP on *in vitro* plant organogenesis has been reported by Lamattina (16). Despite the fact that the individual role of auxin and SNP is well-established in plants, little research has been performed on the effect of individual NO as well as NO + PGRs on *in vitro* shoot regeneration of *Lycopersicon esculentum*.

The present study was undertaken to determine the effects of SNP and plant growth regulators on shoot regeneration and plantlet development in *L. esculentum* Mill. Our literature showed this is the first report on the use of SNP at in vitro cultures of *L. esculentum* Mill

MATERIALS AND METHODS

Optimization of PGRs for shoot induction

Two tomato genotypes, 'Keshtiban' and 'Moneymaker' were kindly provided by the West Azerbaijan Agricultural and Natural Resources Research and Training Center. The seeds were sterilized in the laminar airflow cabinet for 15 minutes with sodium hypochlorite (2.5 %) and were rinsed three times with sterile distilled water. Consequently, the seeds were transferred to a MS medium containing 100 mg L^{-1} myoinositol and 30 g L^{-1} sucrose for germination (Fig. 1A). Cotyledons were cultured on the MS medium containing various combinations of BAP, IAA, IBA and NAA from 7-day-old germinated seedlings (Table 1). The medium pH was adjusted to 5.8 and they were autoclaved at 121C for 15 minutes at 15 psi.

Effect of PGRs on regenerated shoots plants morphology

To develop the regenerated shoots, the MS medium containing different levels of BAP (0.5, 1 and 1.5 mg L^{-1}) and IAA (0.1 and 0.2 mg L^{-1}) was used to culture the shoot buds. Four pieces of cotyledons, with regenerated buds were transferred to shoot elongation medium.

Effect of SNP on shoot regeneration, elongation and leave number per shoot

To determine the impact of SNP in shoot induction and elongation of tomato, a second experiment was carried out. The explants were transferred to MS medium with 2 mg L^{-1} BAP and 0.2 mg L^{-1} IAA (based on the first experiment) supplemented with 5, 10 and 15 µM of SNP. The shoots were transferred onto fresh medium every two weeks. The shoots of 1/5 5 cm were cut from regenerated plants and transferred to the new medium including 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} IAA supplementing with 5, 10 and 15 µM SNP.

Induction of roots and hardening of the plantlets

When the shoots were about 2-3 cm long, they were divided into three groups being transferred to three new culture media: 1. MS medium without PGRs, 2. MS Medium supplemented with IAA (0.2 mg L^{-1}), 3. MS medium with a combination of IAA (0.2 mg L^{-1}) and SNP (10 µM). Following one month of the treatment, the frequency of shoots forming root and root morphology were evaluated. Afterwards, the root-bearing plantlets were removed from the media and rinsed with distilled water to remove any remaining agar. Initially, the plantlets were treated with 20 ppm of bavistin solution for 10 min to prohibit microbial infection. They were then moved to poly-cups containing sterilized coco-peat and were held for ten days under the poly-tunnel. In order to acclimatize, the plantlets were transferred to the greenhouse. Finally, the hardened plants were moved for further growth to the open field.

All cultures were transferred to a plant growth chamber with light to a dark period of 16 h to 8 h, the temperature of 25 ± 2 C and the light of 2500 lux.

Experiments and data analyzing investigation

The whole experiments were carried out with four replications in completely randomized design (CRD). Shoot number per explant, rate of shoot regeneration, stem length and leave number per regenerated shoots were recorded around a month after the experiment was established. The data were further analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($p < 0.05$) with the aid of SPSS (version 17, Chicago, USA) statistical package program. $p < 0.05$ was considered as level of significance.

RESULTS

The optimal PGRs for Shoot regeneration

On the basal MS medium excluding any PGR, no regeneration was detected in cotyledons. The maximum shoot number per explant occurred in Moneymaker (6.75) with a maximum percentage of regeneration (84%) on medium complemented by 2 mg L^{-1} BAP and 0.2 mg L^{-1} IAA, MS medium supplemented with 1 mg L^{-1} BAP showed minimum shoot number per explant (2.25) and shoot induction (28%) (Fig. 1B, C). The highest massive regeneration was observed in the medium containing MS medium with 2 mg L^{-1} BAP and 0.2 mg L^{-1} IAA in the Keshtiban (5 shoot number per explant) at 62%, while the minimum regeneration (2 shoot number per explant) was 25% in MS + BAP 1 mg L^{-1} (Table1).

To get maximum shoot regeneration, the explants were sub-cultured every 15-20 days. Plant regeneration is induced by BAP alone, while regeneration is significantly increased by the combination of BAP and IAA. The combination of BAP and NAA on the regenerations of shoots showed their negative impact on the tomato multiplication rate compared to the individual use of cytokinin (Table 1).

The effect of PGRs on the stem length and leaf number per shoot

On the MS medium with 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} IAA, the highest stem length of 4.6 and 3.4 cm and the highest average of leavesnumber as 7 and 6.6 cm were recorded in Moneymaker and Keshtiban cultivars, respectively (Table 2).

Effect of SNP on adventitious shoot regeneration, stem length and leaf number

The positive impact of SNP on the adventitious shoot regeneration was significant (Fig. 2). Rapid regeneration was achieved in media supplemented with SNP. Under optimal conditions, explants-initiated shoot regeneration within 14 days of initial culture but in SNP supplemented media shoot regeneration occurred only after 10 days. We

Table 1. Effect of different levels of PGRs on shoot number and shoot regeneration frequency from cotyledon after one month of culturing.

PGRs concentration (mg L^{-1})				Explants			
				Keshtiban		Moneymaker	
BAP	IAA	IBA	NAA	shoot number/explants (mean ± SD)	Regeneration percentage	shoot number/explants (mean ± SD)	Regeneration percentage
1	0.0	0.0	0.0	2 ± 0.70^{d}	25%	2.25 ± 0.62^{e}	28%
1.5	0.0	0.0	0.0	2.5 ± 0.50^{bcd}	31%	3 ± 0.57^{de}	37%
2	0.0	0.0	0.0	3.5 ± 0.64^{abcd}	43%	4.25 ± 0.25^{bcd}	53%
1	0.2	0.0	0.0	3 ± 0.70^{bcd}	37%	3.50 ± 0.64^{cde}	43%
1	0.0	0.2	0.0	2.5 ± 0.28^{bcd}	31%	2.75 ± 0.25^{de}	34%
1	0.0	0.0	0.2	2.25 ± 0.25^{cd}	28%	2.50 ± 0.28^{e}	31%
1.5	0.2	0.0	0.0	3.75 ± 0.25^{abc}	46%	4.25 ± 0.47^{bcd}	53%
1.5	0.0	0.2	0.0	3 ± 0.40^{bcd}	37%	3.25 ± 0.47^{de}	40%
1.5	0.0	0.0	0.2	2.5 ± 0.28^{bcd}	31%	3 ± 0.40^{de}	37%
2	0.2	0.0	0.0	5 ± 0.40^{a}	62%	6.75 ± 0.62^{a}	84%
2	0.0	0.2	0.0	4 ± 0.70^{ab}	50%	5.5 ± 0.28^{ab}	68%
2	0.0	0.0	0.2	3 ± 0.40^{bcd}	37%	4.75 ± 0.25^{bc}	59%

The results of twice experiments each with three replicates, each replicate containing four leaf explants. Different letters in a column indicate a significant difference at $p < 0.05$ with Duncan's multiple range test.

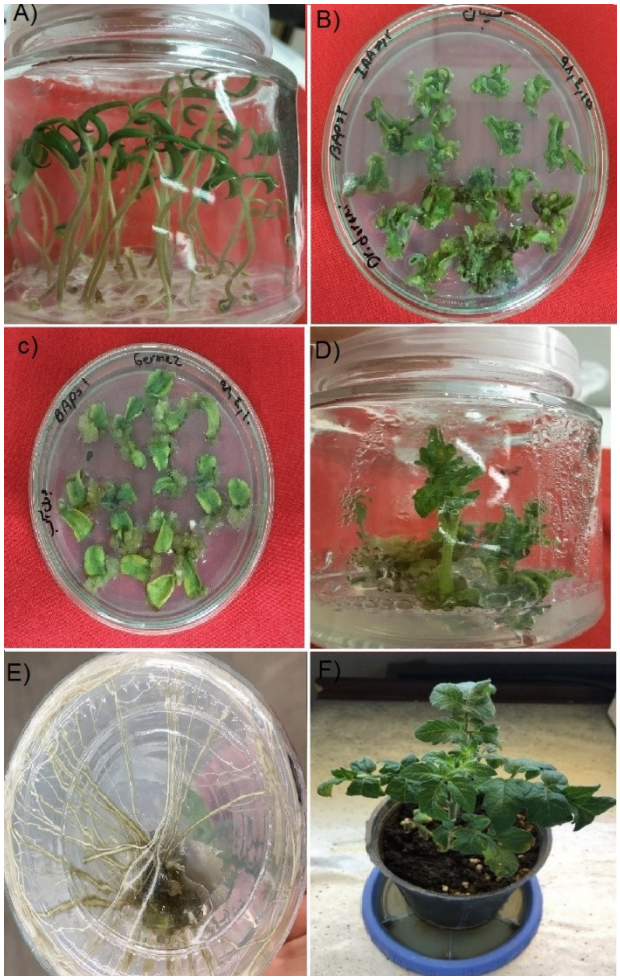

Figure 1. Regeneration of *Lycopersicon esculentum*. A) Seedlings that are 7 days old and have been grown at half strength MS media; B) shoots per explant: MS medium with 2 mg L^{-1} BAP and 0.2 mg L^{-1} IAA; C) MS medium with 1mg L^{-1} BAP; D) Regenerated shoots on elongation medium (MS + 0.5 mg L^{-1} BAP + 0.2 mg L^{-1} IAA); E) Root initiation in MS + 0.2 mg L^{-1} IAA medium; F) Plant acclimatization in soil.

observed that shoot regeneration differed substantially in relevance to the cultivar and SNP levels. Adventitious shoots were promoted in all the media augmented with different concentrations of SNP. The shoot number, as well as regeneration frequency, was greater in all SNP-containing media as opposed to those in SNP-free media. However, the high level of sodium nitroprusside had a negative effect on the shoot organogenesis and shoot number. In the medium containing 10 µM SNP, the maximum number of shoots per explant was obtained. The greatest number of regenerations (as 93% and 90%) and shoots per explant (7.75 and 7.25) were observed in Moneymaker and Keshtiban, respectively (Table 3).

Shoots cultured on MS medium containing 10 µM SNP had a maximum leaf number in Moneymaker (11.2) and Keshtiban (10.2). The highest stem length of 11.8 cm and 10.6 cm were observed in the media supplemented with 10 µM SNP for Moneymaker and keshtiban, respectively (Table 3).

Root initiation and acclimatization

Root formation was initiated within 15 days of cultivation and a well-developed root system was established within one month (Fig.1E). The maximum rooting (88.35% and 87.69%) occurred in MS medium supplemented with 0.2 mg L^{-1} IAA and SNP 10 for Moneymaker and Keshtiban, respectively (Table 4).

It was found that fewer adventitious roots and higher lateral roots were significantly developed in the MS medium containing IAA and SNP. Lateral roots were distributed at the lower and upper parts of the adventitious root (Fig. 3A, 3B). Increased adventitious roots without

Table 2. The effect of applied PGRs concentrations on stem length and leaf number per shoot after two months of culturing

PGRs concentration (mg L^{-1})		Explants			
		Keshtiban		Moneymaker	
BAP	IAA	Stem length (cm) (mean ± SD)	Leaf number (mean ± SD)	Stem length (cm) (mean ± SD)	Leaf number (mean ± SD)
0.5	0.0	2.4 ± 0.40^{ab}	4.8 ± 0.66^{b}	2.6 ± 0.24^{bc}	4.6 ± 0.50^{bc}
1	0.0	2 ± 0.44^{b}	4 ± 0.31^{bc}	2.2 ± 0.37^{bc}	4.4 ± 0.24^{c}
1.5	0.0	1.8 ± 0.37^{b}	2.8 ± 0.37^{cd}	1.8 ± 0.37^{c}	2.4 ± 0.50^{d}
0.5	0.1	2.6 ± 0.24^{ab}	5.2 ± 0.37^{b}	3 ± 0.31^{b}	6 ± 0.44^{ab}
1	0.1	2.2 ± 0.58^{ab}	3 ± 0.44^{cd}	2.6 ± 0.40^{bc}	5.2 ± 0.58^{bc}
1.5	0.1	1.8 ± 0.37^{b}	2 ± 0.31^{d}	2 ± 0.31^{bc}	2.6 ± 0.40^{d}
0.5	0.2	3.4 ± 0.50^{a}	6.6 ± 0.50^{a}	4.6 ± 0.40^{a}	7 ± 0.70^{a}
1	0.2	2.8 ± 0.20^{ab}	5 ± 0.54^{b}	3 ± 0.31^{b}	5.4 ± 0.50^{bc}
1.5	0.2	2.4 ± 0.24^{ab}	3.8 ± 0.37^{bc}	2.8 ± 0.20^{bc}	4.2 ± 0.37^{c}

The results of twice experiments each with three replicates, each replicate containing four explants. Different letters in a column indicate a significant difference at $p < 0.05$ with Duncan's multiple range test.

lateral roots were resulted by the medium containing IAA alone (Fig. 3C). Shoots rooted in the presence of SNP resulted in healthy plantlets within 30 days of culture with improved root (Root initiation frequency and root length) compared to the control.

Figure 2. Effects of different SNP concentrations on the regeneration of adventitious shoots A) 0 µM; B) 5µM; C) 10µM; D) 15µM.

Figure 3. Rooted plantlets on A) MS medium without PGRs; B and C) MS + IAA 0.2 mg L^{-1} and SNP 10 µM; D) MS + IAA 0.2 mg L^{-1}.

Table 3. SNP effect on shoot number per explants, frequency of shoot regeneration, the stem length and leaf number from cotyledon after one month from culture

PGRs (mg L^{-1})		SNP µM	Explants								
			Keshtiban					Moneymaker			
BAP	IAA		Shoot number/explant (mean ± SD)	Regeneration frequency	Mean of stem length (cm)	Mean of leaf number	shoot number/explant (mean ± SD)	Regeneration frequency	Mean of stem length (cm)	Mean of leaf number
2	0.2	0.0	4.50±0.47^{b}	60%	-	-	6.25±0.64^{b}	78%	-	-
2	0.2	5	5.5±0.64^{ab}	68%	-	-	6.75±0.25^{ab}	81%	-	-
2	0.2	10	7.25±0.47^{a}	90%	-	-	7.75±0.50^{a}	93%	-	-
2	0.2	15	5.25±0.47^{b}	65%	-	-	6.50±0.22^{b}	79%	-	-
0.5	0.2	0.0	-	-	2.8±0.20^{c}	6.6±0.50^{b}	-	-	4.2±0.37^{c}	6.2±0.37^{c}
0.5	0.2	5	-	-	5.8±0.37^{b}	8.4±0.81^{ab}	-	-	6.6±0.50^{b}	9.4±0.50^{b}
0.5	0.2	10	-	-	10.6±0.67^{a}	10.2±0.66^{a}	-	-	11.8±0.58^{a}	11.2±0.86^{a}
0.5	0.2	15	-	-	5.0±0.70^{b}	7.6±0.92^{b}	-	-	5.6±0.24^{b}	7.4±0.50^{c}

The results of twice experiments each with three replicates, each replicate containing four leaf explants. Different letters in a column indicate a significant difference at $p < 0.05$ with Duncan's multiple range test.

Table 4. Root initiation frequency on MS media containing various levels of IAA and SNP.

IAA (mg L^{-1})	SNP (µM)	Explants	
		Root initiation Keshtiban (mean ± SD)	Root initiation Moneymaker (mean ± SD)
0.0	0.0	26.67±0.58^{c}	29.20±0.22^{c}
0.2	0.0	45.02 ±0.20^{bc}	50.40 ±0.49^{bc}
0.2	10	87.69±0.53^{a}	88.35±0.30^{a}

The results of twice experiments each with three replicates, each replicate containing four explants. Different letters in a column indicate a significant difference at $p < 0.05$ with Duncan's multiple range test.

DISCUSSION

The adverse effect of auxin on the shoot induction in mung bean (17), faba bean (18) and cotton (19) has been reported. Based on the literature, the application of NAA in cytokinin-containing media has not enhanced the rate of multiplication of the shoots. In agreement with our observation, Rai et al. (20) reported formal induction of multiple shoots of tomato on MS medium supplemented with 2.0 mg L^{-1} BAP + 0.2 mg L^{-1} IAA. Similar findings were also reported by BAP and IAA in tomato (21). MS medium with 6.65 µM BAP and1.14 µM IAA has been introduced as the best media for shoot induction of cotyledon explants (22). The positive synergic influence of cytokinins and auxins on direct organogenesis in tomato might be originated from the modification of physiological processes and morphogenic responses. Gerszberg et al. (23) stated that the best regenerative response was obtained with MS medium augmented with 2 mg L^{-1} BAP and 0.1 mg L^{-1} IAA in *Solanum lycopersicum*.

According to a report, nitric oxide can be produced in suspension cell cultures treated with BAP at a proper dose (24). Plant growth regulators were found to be fully correlated with NO's effect on plant production and growth (16).

SNP addition significantly improved the morphology characteristics, the data revealed that adding 10 µM SNP to tomato regeneration medium improved plant growth and increased shoot regeneration frequency (stem length and leave number). This point suggests that the method of the applied culture might be an appropriate strategy for mass propagation for commercial purposes (25).

SNP can induce cell wall relaxation by its function on the lipid bilayer of the cell membrane, resulting in plant development and cell expansion. Furthermore, as auxins induce their biosynthesis, the SNP effect is generated by the mediation of ethylene (24). *Valeriana jatamansi* Jones had the strongest response in the medium containing 10% coconut water + 15 µM SNP as 89.32% (25). In chrysanthemum, it has been found that the medium containing SNP, 2,4-D and KIN formed the most somatic embryos per explant (10). In *Tagetes erecta*, MS medium containing BAP, IBA, and 30 µM SNP resulted in the maximal shoot number and shoot organogenesis frequency from callus (24), whilst MS medium containing 60 µM SNP showed the highest root formation (100%) and root numbers. In chrysanthemum cultivars, combining BAP and SNP (as composed to the use of only BAP) improved explant morphogenetic capacity and increased shoot regeneration (16).

While rooting takes place in the media augmented with IAA, IBA, and NAA media, many researchers have confirmed that IAA is the most preferred rooting hormone for tomato. IAA is used for rooting in full strength MS media or media with changed MS salts and these findings were consistent with the current results (26).

CONCLUSION

The potential of successful genetic manipulation in plants is largely dependent on the availability of reliable regeneration systems. Tomato is the most commonly used vegetable crop for genetic engineering. To meet the high commercial demand and to conserve this plant, it is critical to optimize an efficient protocol for *in vitro* propagation. Supplementation of SNP had a substantial effect on *in vitro* shoot regeneration and plantlet development in *Lycopersicon esculentum* Mill. According to the results of this study, the combination of 2 mg L^{-1} BAP + 0.2 mg L^{-1} IAA + 10 µM SNP, as a quick and efficient protocol for shoot regeneration and 0.5 mg L^{-1} BAP and 0.2 mg L^{-1} IAA + 10 µM SNP for the stem length and leaf number per shoot and 0.2 mg L^{-1} IAA + 10 µM SNP for the root initiation and acclimatization were developed. Thus, we believe that the improved tissue culture methodology presented in this study, which included SNP, will provide a viable technique for *in vitro* propagation of this species.

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تأثیر سدیم نیتروپروساید (SNP) و تنظیم‌کننده‌های رشد گیاهی بر باززایی ساقه و رشد گیاهچه *Lycopersicon esculentum* Mill. در

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چکیده

بهینه‌سازی شرایط باززایی گیاهان در مطالعات کشت بافت نه‌تنها به دلیل ارزش اقتصادی محصولات تجاری، بلکه برای گزارش‌های علمی نیز ضروری است. در این مطالعه باززایی و رشد گیاهچه. *Lycopersicon esculentum* Mill با استفاده از غلظت‌های بهینه تنظیم کننده‌های رشد گیاهی که با سدیم نیتروپروساید تکمیل شده بودند، بهبود یافتند. با توجه به نتایج، از بین ۱۲ ترکیب مختلف تنظیم‌کننده رشد گیاهی استفاده شده، محیط MS تکمیل‌شده با ۲ میلی‌گرم در لیتر BAP و ۰/۲ میلی‌گرم در لیتر IAA بیشترین درصد باززایی (۸۴ درصد) را داشت. بیشترین طول ساقه و تعداد برگ در محیط کشت MS حاوی ۰/۵ میلی‌گرم در لیتر BAP و ۰/۲ میلی گرم در لیتر IAA ثبت شد. افزودن ۱۰ میکرومولار سدیم نیتروپروساید به محیط کشت، درصد باززایی (۹۳ درصد) را بهبود بخشید. علاوه بر این، بالاترین طول ساقه و بیشترین تعداد برگ، در محیط MS حاوی ۰/۵ میلی گرم در لیتر BAP و ۰/۲ میلی گرم در لیتر IAA و ۱۰ میکرومولار سدیم نیتروپروساید به دست آمد. در محیط MS حاوی IAA و سدیم نیتروپروساید ریشه‌های فرعی کمتر و ریشه‌های جانبی بیشتری تولید شد، ریشه‌های جانبی در قسمت‌های تحتانی و بالایی ریشه فرعی توزیع شد. نتایج این پژوهش نشان داد که افزودن SNP به محیط کشت .*Lycopersicon esculentum* Mill باززایی شاخه و رشد گیاه را بهبود بخشید که این امر احتمالاً بر مشکلات تکثیر و همچنین اصلاح ژنتیکی گیاه گوجه‌فرنگی غلبه کند.

کلمات کلیدی: سدیم نیتروپروساید، تنظیم کننده‌های رشد، باززایی، گوجه‌فرنگی