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Original Article

Investigation Effects of Some Plant Hormones and Vitamins on Growth in Kanamycin-Resistant Tomato Varieties

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ABSTRACT

Tomato is a widely consumed vegetable around the world and has a significant economic impact on agricultural production. However, various challenges in current agricultural practices can affect tomato production. To overcome these challenges and create a sustainable agricultural model, research focusing on tomato plant tissue culture is gaining importance. In this study, the neomycin phosphotransferase (NPTII) reporter gene was transferred to the Lycopersicon esculentum cLN1558A line through *A. tumefaciens* LBA4404. The effects of phenolic compounds and growth regulators used in tomato tissue culture were investigated. A balanced concentration of 1 mg ml^{-1} 6-benzyladenine (BA) and 1 mg ml^{-1} gibberellic acid (GA_3) was used for optimal shoot regeneration. For optimal root regeneration, 1 mg ml^{-1} indole-3-acetic acid (IAA) concentration was used. 200 µM acetosyringone was added to the culture medium to accelerate *A. tumefaciens* infection. As a result, it was confirmed that kanamycin resistance reached 50% in transgenic tomato shoots containing the NPTII gene. The efficiency of the phenolic compounds and growth regulators used increased by 12%.

Key words: Giberellic acid, 6-benzyladenine, NPTII, tomato tissue culture

Kanamisine Dayanıklı Domates Çeşitlerinde Bazı Bitki Hormonları ve Vitaminlerinin Büyümeye Etkisi

ÖZ

Domates dünya çapında yaygın olarak tüketilen bir sebzedir ve tarımsal üretim üzerinde önemli bir ekonomik etkiye sahiptir. Ancak mevcut tarımsal uygulamalardaki çeşitli zorluklar domates üretimini etkileyebilmektedir. Bu zorlukların üstesinden gelmek ve sürdürülebilir bir tarım modeli oluşturmak için domates bitkisi doku kültürüne odaklanan araştırmalar önem kazanmaktadır. Bu çalışmada neomisin fosfotransferaz (NPTII) raportör geni, A. tumefaciens LBA4404 aracılığıyla *Lycopersicon esculentum* cLN1558A hattına aktarıldı. Domates doku kültüründe kullanılan fenolik bileşiklerin ve büyüme düzenleyicilerin etkileri araştırıldı. Optimum sürgün rejenerasyonu için dengeli bir 1 mg ml^{-1} 6-benziladenin (BA) ve 1 mg ml^{-1} gibberellik asit (GA3) konsantrasyonu kullanıldı. Optimum kök rejenerasyonu için 1 mg ml^{-1} indol-3-asetik asit (IAA) konsantrasyonu kullanıldı. *A. tumefaciens* enfeksiyonunu hızlandırmak için kültür ortamına 200 µM asetosiringon eklendi. Sonuç olarak NPTII genini içeren transgenik domates sürgünlerinde kanamisin direncinin %50'ye ulaştığı doğrulandı. Kullanılan fenolik bileşiklerin ve büyüme düzenleyicilerin etkinliği %12 artmıştır.

Anahtar kelimeler: Giberellik asit, 6-benzyladenine, NPTII, domates doku kültürü

INTRODUCTION

Transgenic crops are bred for a variety of purposes: to be resistant to fungal, bacterial, viral diseases and insect pests, to tolerate herbicides and to grow better under restricting environmental conditions such as drought, high salt or metal containing soils. Increasingly, the improvement of quality traits such as nutritional content and suitability for industrial processing is also of importance. Essentially, conventional breeding has very

similar goals such as to develop plant varieties with higher yield and/or better nutritional qualities, disease and pest resistance and lower cultivation costs. Agrobacterium-mediated tomato transformation has developed rapidly over the last 50 years. Many factors such as plant diversity, plant material used, growth regulators, and bacterial species can change the effect of transformation (Ellul et al., 2003; Ohki, Bigot, & Mousseau, 1978; Shahin, Sukhapinda, Simpson, & Spivey, 1986).

Tomato (*Solanum lycopersicum L.)* is a perennial plant in tropical regions, containing lycopene and various minerals and vitamins, while it is an annual cultivated plant in other regions (GaffaroĞLu, Horuz, & Aysan, 2019). Some phenolic molecules secreted by plant cells accelerate Agrobacterium transformation by inducing the expression of vir genes (Bolton, Nester, & Gordon, 1986; Stachel, Nester, & Zambryski, 1986). In some plants that cannot produce these phenolic molecules, the use of acetosyringone increases the transformation efficiency by inducing genes (Sheikholeslam & Weeks, 1987). In addition, different phenolic compounds obtained from plants such as tobacco can be used as transformation inducers (Hamza & Chupeau, 1993; Kaya, Al-Remİ, Arvas, & DurmuŞ, 2018). Indole-acetic acid (IAA), used in plant tissue culture, is important for many plants, especially tomato plants. Strong callus size and organogenesis occur in the IAA plant (Magnus, Hangarter, & Good, 1992; Ohki et al., 1978; Pfitzner, 1998). α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BA) used in tomato tissue culture enable shoot formation in the hypocotic segments of the plant (Yakuwa T, 1973). Additionally, application of some humic substances (Bio Humate, Justyne) to tomato seedlings increases the vegetation in the plant (KizildenİZ, VacelİK, & Bettonİ, 2022). It has been revealed that the combined use of these hormones in plant tissue culture studies provides benefits such as shoot differentiation and shoot formation from hypocotyl and leaf segments (Ohki et al., 1978). Additionally, some phenolic compounds secreted by active plant may facilitate T-DNA transfer by increasing the vir gene expression of *A. tumefaciens* (Janssens, Genetello, Van Montagu, & Zambryski, 1986). A study revealed that the use of BA alone or in combination with 0.75 mg/L PBZ induced in vitro flowering in tomatoes (Dewi, Prasetyo, Sukweenadhi, Irawati, & Savitri, 2022). However, the combination of IAA and cytokinins BA, 6(γγ-dimethyl-allylamino)-purine (IPA) and kinetin on shoot differentiation medium gives the best results in shoot formation from the hypocotyl and leaf segments of IPA (Ohki et al., 1978). The use of different plant bodies may produce different effects in transformation. From past to present, different plant parts, including cotyledons, hypocotyls, and stem parts, have been used in tissue culture-mediated transformation (Chyi & Phillips, 1987; Pfitzner, 1998).

This study aims to reveal the effect of 6-benzyladenine (BA) and gibberellic acid (GA3) concentration and acetosyringone induction on explant and shoot regeneration and to modify the tomato-specific protocol with Agrobacterium transformation.

MATERIALS AND METHOD

Plant Regeneration

Commercial *Lycopersicon esculentum cv*. seeds were sterilized in 70% ethanol for 3 min and in 30% chlorax for 7 min. Seeds were placed in Murashi-Skoog (MS) medium, 5 in each petri dish, and germinated at 8/16 photoperiod. Cotyledons were cut from tomato seedlings and their ends were cut transversely into two pieces. Cotyledons were placed in petri dishes (90x15 mm) containing preculture medium and incubated in the dark at 25 °C, 70% humidity for 2 days (Table 1).

Construction of Vector

NPTII gene were amplified by polymerase chain reaction (PCR) using the full-length NPTII genomic clones. The constructs of pBI121-NPTII amplified in the plasmid pBI121 as a template. PCR was performed in a 50 µl reaction solution containing 0.3 µg template DNA (pBI121-NPTII), 20 nM of each primer, 0.05 mM dNTPs, 0.125 µl LA Taq (Takara, Japan), 2.5 mM MgCl2, 10X LA PCR buffer (Mg +2 free).

All of the ligation products were transformed into *Escherichia coli* DH5α using the heat-shock method. One hundred µl of the component *E. coli* cell was added to the ligation product (20 µl), put on ice for 30 minutes and followed by heat shock in the water bath at 42 °C for 1 minute, and immediately put on ice for 3 minutes and 1 ml of LB Broth was added. After 1 hr incubation at 37 °C, it was centrifuged at 10000 rpm for 3 minutes and the supernatant was discarded. The pellet was cultivated in LA medium containing 50 µg/ml spectinomycin and incubated at 37 °C. *E. coli* containing the target plasmid were confirmed by colony PCR. Individual colonies were used for PCR. The pBI121 plasmids DNA of the transformants were isolated from E. coli using by plasmid miniprep purification kit (Macherey Nagel., Düren, Germany). Briefly, 4 ml of *E. coli* culture were centrifuged 12000 rpm for 1 minute and the supernatant was removed before solution I (200 µl) was added to remove the RNAs. Solution II (200 µl) was added to wash the proteins. Solution III (200 µl) was added to wash off the other ingredients before centrifugation at 12000 rpm for 5 minutes. The supernatant was transferred to the new spin

column and centrifuged at 12000 rpm for 1 minute, and the lower tube was removed and centrifuged for 1 minute at 12000 rpm by adding 700 µl of wash solution. The column was attached to the new tube and placed in the oven for 3 minutes and 100 µl of dH2O was added and again put in the oven for 10 minutes and centrifuged at 12000 rpm for 1 minute. The accuracy of the plasmid isolated from E. coli was confirmed by gel electrophoresis. The isolated plasmid was stored at -20 ºC by discarding the column.

The pBI121 plasmid including target NPTII was transformed into Agrobacterium tumefaciens LBA4404 using by a GenePulser II electroporator (Bio-Rad, California, USA). Briefly, A. tumefaciens LBA4404 was cultured in LB medium for 2 days at 28 °C and single colony was selected and transferred to 5 ml of LB broth and left overnight. Then 100 µl of the culture was subcultured and incubated at 28 °C for 6-7 hours and then left on ice for 15 minutes. It was centrifuged at 5000 rpm for 20 minutes and the pellet was washed with 10 ml of 1mM HEPES (4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid) and the same solution was thawed with 10 ml of HEPES and centrifuged at 5000 rpm for 15 minutes and the supernatant discarded. The pellet was dissolved in 750 µl 10% glycerol and an aliquot was pipetted into 45 µl tubes and stored at -80 °C. Forty-five µl of the component cell was added to the agrobacterium culture, 1 µl of the isolated plasmid was transferred to the electroporation cuvette and electroporated, and then 1 ml of SOC was added to the whole solution and transferred to the new Eppendorf tube and spread to the LA medium containing streptomycin (50 µg) and spectinomycin (50 µg) antibiotics and cultured at 28 °C. The accuracy of agrobacterium was confirmed by colony PCR.

Plant Transformation

A. tumefaciens LBA4404 harboring pBI121 carrying the NPTII gene was grown in LB medium and OD600 was maintained at 0.8. Tomato cotyledons were kept in A. tumefaciens bacterial suspension for 30 min. Cotyledon explants were placed in equal numbers in culture medium PLa; containing only MS and Agar, PLb; containing 200 µM acetosyringone, in PLc; containing BA, AS, and PLd; containing GA_3, AS kept at 25 °C for 2 days (Table 1). After incubation in the growth chamber, dried disks of sterile filter paper were placed on them and cotyledon explants were then placed on the filter paper. After 2 days, cotyledons were transferred to co-culture containing the same vitamins and incubated in a photoperiod of 8/16 hours. The plants, which reached a height of 2-3 cm after 3 weeks of incubation, were transferred to the rooting medium and rooted.

Table 1. Compositon of culture media

	PL[a]	**PL[b]**	**PL[c]**	**PL[d]**
MS[a]	+	+	+	+
Sucrose (g L^{-1})	30	30	30	30
Agar (g L^{-1})	8	8	8	8
Acetosyringone (mM)		200	200	200
BA (mg L^{-1})			1	
GA_3 (mg L^{-1})				1
IAA (mg L^{-1})			0.5	0.5
Kanamycin (mg L^{-1})	100	100	100	100

MS[a]: Murisime and Skoog (1962), GA_3: Giberellic acid, BA: 6-benzylaminopurine, IAA: Indole-asetic acid, PL[a]: Pre-culture medium, PL[b]: Co-culture medium, PL[c]: Regeneration medium, PL[d]: Rooting medium.

Genetic Analysis

Expression of the NPTII gene was detected in T1 tomato plants obtained from T0 plant. After the obtained seeds were sterilized and germinated in PLA media. It was transferred to PLb media containing 100 mg l^{-1} kanamycin. Rooted plants were scored for kanamycin resistance after 3-4 weeks of culture. Wild-type tomato leaves lacking the NPTII gene did not grow on medium containing kanamycin.

Molecular Analysis

Total DNA samples were extracted from the samples of tomato to determine the presence of the NPTII gene. Total DNA was isolated from tomato tissue (Doyle, 1987) and the isolated was used in PCR for the detection of NPTII gene. PCR analysis was performed using Taq OptiMix CLEAR 2x Master Mix® (Ampliqon, Odense, Denmark). The following gene specific primer was used (Cortina & Culiáñez-Macià, 2004). The amplicification conditions for the PCR reaction was as follows 95 °C for 5 min, 94 °C for 30 s, 59 °C for 30 s and 72 °C for 30 s for 35 cycles; 5 min for 72 °C and for each 25 µl sample mixture. The data was analyzed by the electrophoresis method.

RESULTS AND DISCUSSION

Effect of BA on tomato explants survival

The effect of BA concentration in the shoot regeneration medium is shown in Figure 1. Callus formation was observed from tomato leaves on MS medium (PLc) containing BA. However, no callus formation was observed in tomato leaves on MS medium that did not contain BA, which was used as a negative control.

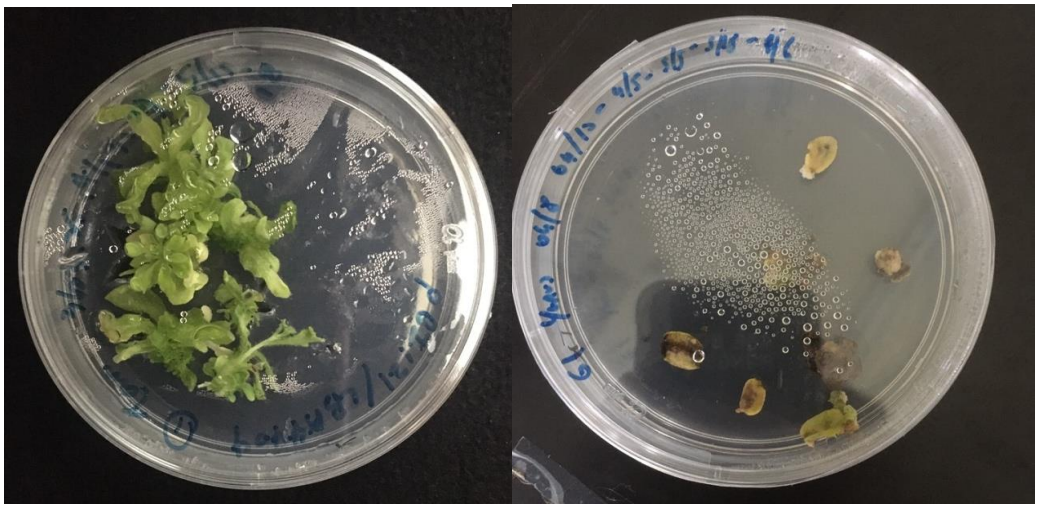

Figure 1. BA effect on cotyledon explants survival. Cotyledon explants on MS regeneration medium containing 0,1 mg l^{-1} BA PLc (left) and on MS modified regeneration medium does not containing BA (right), after 14 days.

Effect of GA_3 on tomato explants survival

The effect of using GA_3 on rooting can be seen in Figure 2. Whereas on medium does not contain GA_3 no more than 8% of shoots where obtained, on medium with 0.1 mg l^{-1} GA_3 PLd the regeneration rate was three times higher (25%).

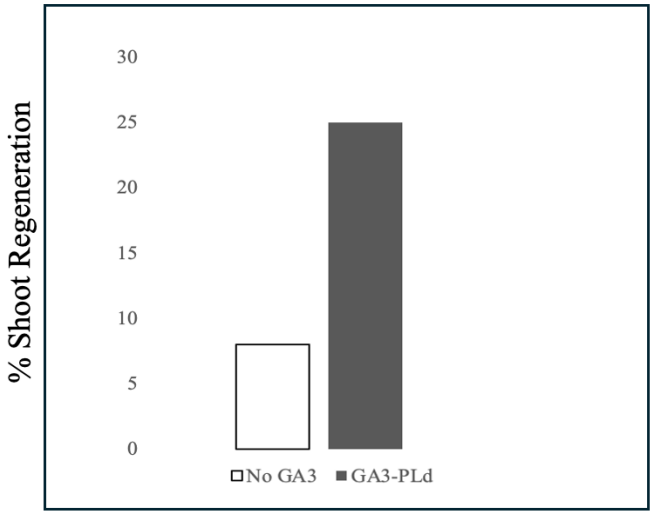

Figure 2. Effect of GA_3 on shoot regeneration medium. Percentage of resistant shoots per explant on medium containing 0,5 mg l^{-1} IAA (white column) (negative control) and on medium containing 0.5 mg l^{-1} BA and 0.5 mg l^{-1} IAA PLd (black column). Data represent the mean value of two different experiments (100 cotyledon explants for each transformation procedure) ± SD. Values were significantly different (T Student distribution for $p < 0.05$).

Acetosyringone effect on transgenic plant recovery

The effect of acetosyringone added to PLc and PLd broth media is seen in Figure 3. Certain ratios are considered optimal for the use of acetosyringone in the production of T-strands. Therefore, the same amount of acetosyringone (200 µm) was added to both media. The plant transformation rate (PLc) from this treatment, as

the number of confirmed transgenic plants per shoot, was over 50%; this was four times higher than that obtained with PLd medium (11%).

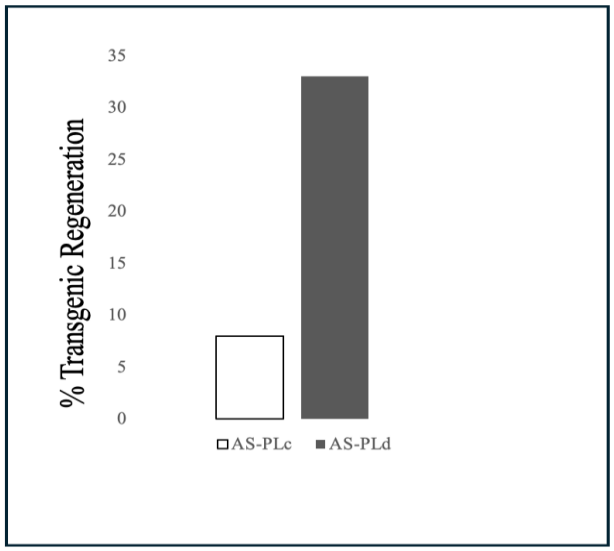

Figure 3. Effect of acetosyringone on transgenic plant recovery. Percentage transgenic plants per shoot on medium containing 200 µM acetosiryngone PLc (Table 1) (white column), and on medium containing 200 µM acetosiryngone PLd (Table 1) (black column). Data represent the mean value of two independent experiments (100 cotyledon explants for each trans- formation experiment) ± SD. Values were significantly different (T Student distribution for $p < 0.05$).

Molecular analysis of T0 transgenic plants

DNA from wild type and randomly selected T0 independent transformed cLN1558A tomato plants was PCR amplified using 5 -forward and 3 -reverse NPTII gene primers (Figure 4). A band of about 1.1 kb corresponding to the predicted size of the gene fragment, confirmed the T-DNA integration in the genome of these plants.

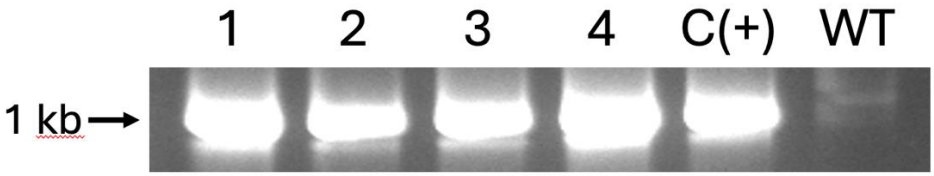

Figure 4. Molecular analysis of transgenic plants. Genomic DNA from Lycopersicon esculentum cv. cLN1558A wild type (WT) and T0 transformed plants, PCR amplified using specific NPTII gene primers. C (+): positive control of pBI121 plasmid. The left arrow indicates the band molecular weight.

Genetic Analysis

Tomato plants were self-pollinated to analyze the inheritance of kanamycin resistance in 4 random plants including NPTII gene. Seeds were collected from pollinated plants and germinated in PLa medium containing 100 mg l^{-1} kanamycin. After 3-4 weeks, leaf and root development were distinguished from susceptible seedlings lacking the NPTII gene. This heritability analysis is included in Table 2.

Table 2. Inheritance of Kanamycin resistance of the T1 generation of independent lines transformed by *A. tumefaciens* LBA4404 harbouring a *NPTII* gene for Kanamycin resistance

Line	Number of T1 seedling		x^2 3:1
	R	S	
WT	0	100	300
1	78	22	0.45*
2	84	16	0.22*
3	75	25	0.34*
4	68	32	2.29*

CONCLUSION

While tomato transformation has become a routine procedure, challenges persist in achieving a satisfactory yield of positive plants from viable calluses. This study presents a simple and effective modification for tomato genetic transformation and regeneration. The widely adopted MS medium (Murashige & Skoog, 1962) serves as the primary commercial medium in tissue culture. Notably, incorporating GA_3 and BA at a concentration of 1 mg l^{-1} in the MS shoot regeneration medium facilitates cell growth and regeneration.

The T-DNA transfer process is predominantly governed by the vir genes (Garfinkel & Nester, 1980). Activation of the vir genes is induced by a family of small phenolic molecules, such as acetosyringone, thereby promoting T-DNA transfer (Bolton et al., 1986). The synthesis of T-strand derivatives from the nicked T-DNA substrate is notably associated with a concentration of 200 µM acetosyringone (Culianez-Macia & Hepburn, 1988). By incorporating this optimal acetosyringone concentration into the co-culture medium, the transformation efficiency experienced a fourfold improvement. Additionally, 50% of the resistant shoots successfully rooted on kanamycin and were subsequently confirmed as transgenic plants. This modification offers a promising approach to enhance the efficiency of tomato genetic transformation and regeneration.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Author Contributions

***1st* Yunus Emre Uslu :**Conceptualization; data curation; methodology; project administration; software; writing—original draft; writing—review and editing.

2st İsmail Bezirganoglu: Formal analysis; funding acquisition; investigation;project administration; software; writing— original draft; writing—review and editing.

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