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IN VITRO MUTAGENESIS AND PROPAGATION OF *PAULOWNIA TOMENTOSA* (THUMB) FOR SALT TOLERANCE

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SUMMARY

The study aimed to refine a protocol for micropropagation and to develop the plant's ability to withstand salinity by the use of physical and chemical mutations, so that it can be cultivated in new lands that are not suitable for other crops. Shoot tips and stem segments of *Paulownia tomentosa* were firstly sterilized and cultured on different media types containing benzyl amino purine (BAP) at 0, 0.1, 0.25, 0.5, and 1.0 mg/l to choose the best combination for explant growth and proliferation. To examine the plant's ability to withstand salinity, *Paulownia tomentosa* shoots were first irradiated with the doses of gamma rays at 0.0, 30, 60, 90, 120, and 150 Gray (Gy) and secondly, cultured on a WPM medium containing sodium azide "NaN_3" at 0.0, 0.1, 0.2, 0.4, 0.8. and 1.0 mM for 5 min. Both irradiated and NaN_3-treated shoots were cultured on different levels of NaCl. Inter Simple Sequence Repeats (ISSR) technique was used to detect variations caused by gamma rays and NaN_3. Results showed that at 120 Gy of gamma-ray, one fragment with primer UBC824 vanished and one fragment with primer 17898B at 150 Gy appeared. In comparison, one fragment with primer either UBC873 or UBC867 at 1.0 mM and 0.8 mM of NaN_3, respectively, can be considered a positive marker of Paulownia salt tolerance. Treated shoots gave the greatest number of roots/shoot (6.0) on WPM half strength with NAA at 2.0 mg/l. Increasing gamma doses or NaN_3 concentrations decreased survival rate. Variation created by mutation provides the raw material for natural selection and is a driving force in evolution.

Keywords: Gamma-ray, mutagenesis, NaN_3, *Paulownia tomentosa,* proliferation, salt tolerance, tissue culture

Key findings: At 120 Gy of gamma-ray, one fragment with primer UBC824 vanished and one fragment with primer 17898B at 150 Gy appeared. In comparison, one fragment with primer either UBC873 or UBC867 at 1.0 mM and 0.8 mM of NaN_3, respectively, and one fragment with primer UBC828 at 0.8 mM of NaN_3 appeared, which can be considered as a positive marker of Paulownia linked to salt tolerance.

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INTRODUCTION

In the last few years, Paulownia (*Paulownia tomentosa* [Thumb.]) has been introduced into Polish nursery production as a new and very prospective plant. *Paulownia* is an empress tree (Barton *et al*., 2007) belonging to the Paulowniaceae family, and is a fast-growing hardwood tree. *Paulownia tomentosa* is the most temperature tolerant than other species. In herbal medicine, *P. tomentosa* extracts are used to treat illnesses, such as, gonorrhea, bronchitis, erysipelas, dysentery, or acute enteritis (Bahri and Bettaieb, 2013). *Paulownia tomentosa* is commonly used as an ornamental plant and can be used for energy production, wood construction materials, and paper pulp production.

Apart from its ornamental value, it is perfect for biomass production and a top-quality wood source. The plant can reach a height of 20 m after six years and measures 35 cm in diameter. Its wood can be used for the production of furniture, doors, window frames, toys, composite boards, or musical instruments. Its timber is light and strong. It dries very easily and is resistant to deformation. From an economic point of view, Paulownia is important for growers as itdoes not need re-planting because it grows from stumps after harvesting, and the process can be repeated many times (Chunchukov and Yancheva, 2015).

Traditional species propagation generally requires large land areas and a large number of human resources, but it can also be done in milder climates and sheltered spaces, ensuring proper caution with its surrounding areas, and complying with required temperatures as favorable isotherms. Paulownia can be propagated from seeds or cuttings using traditional methods. Seed germination is slower compared with root cuttings or branches cultured *in vitro*. Therefore, this is the main reason why effective vegetative reproduction methods must be adopted. In comparison to root cuttings or shoot cuttings derived from *in vitro* cultures, seeds germinate slowly and grow slower (Pożoga *et al*., 2019). The multiplication of species through tissue culture, with the prospect of simultaneous large seedling quantity planting, is characterized by maximum biological homogeneity, soundness, and low cost, regardless of season, climate, and altitude (Bergmann, 1998).

The degradation of the environment causes biotic and abiotic stresses in plants and restricts their production and growth (Shao and Chu, 2005; El-Atawy *et al*., 2021). Salinity and drought are the main abiotic stresses that affect crops worldwide (Vinocur and Altman, 2005) and cause serious harm to plants, including growth inhibition, necrosis, impaired metabolism, development, and quality reduction (Sivritepe and Eris, 1999; Lang *et al*., 2019, 2020). Salinization has continued to rise globally (Rengasamy, 2006) and poses a major threat to land resources, delays plant growth in crop production, and harms the economy and the environment (Rengasamy, 2010; Yang *et al*., 2010). An effective and economical way to use salinized land is to grow salt-tolerant plants. Salinity stress causes oxidative stress to accumulate reactive oxygen species (ROS) (O_2, superoxide radicals, OH, hydroxyl radical, H_2O_2, and hydrogen peroxide). DNA, lipid peroxidation, and enzyme inactivation may be impaired by oxidative stress (Gill and Tuteja, 2010). The Paulownia tree is considered rather salinity-sensitive (Ayala and Alcaraz, 2010; Ivanova *et al*., 2014).

Induced mutagenesis is one of the most efficient tools that has been utilized extensively to create genetic variation and identify key regulatory genes for economically important traits in crop improvement (Chaudhary *et al*., 2019). Mutation breeding and plant mutagenesis play a significant role in increasing the genetic variability for desired traits in various food crops (Adamu and Aliyu, 2007; Mostafa, 2011; Kozgar *et al*., 2012). Induced mutagenesis is one of the most efficient tools used, not only for the identification of key regulatory genes, but also for molecular mechanisms. It is a promising approach for developing new varieties with improved agronomic characteristics, such as, higher stress tolerance potential (biotic and abiotic stress) and bio-fortification.

Traditionally, mutations are caused by treating both seeds and vegetatively propagated crops with physical (gamma radiation) and chemical mutagen. Suitable mutants with useful agronomic characteristics, e.g., biotic and abiotic stress resistance, can be isolated and propagated at higher rates in a short period using *in vitro* propagation (Jain, 2010). The development of efficient tissue culture protocols is required for genetic improvement. Biotechnology advancements have created new opportunities for salinity improvement of *Paulownia tomentosa*. *In vitro* propagation, thus, has proven to be a powerful tool for overcoming issues associated with field culture. The main objective of this investigation was to optimize a protocol for

micropropagation using various plant growth regulators and to develop the plant's ability to withstand salinity by using physical and chemical mutations, so that it can be cultivated in new lands that are not suitable for other crops.

MATERIALS AND METHODS

Between 2018 and2022, the experimental work on *Paulownia tomentosa* was performed at the Tissue Culture Laboratory, Department of Plant Genetic Resources, Desert Research Centre, Egypt, to enhance and evaluate the plant's *in vitro* propagation potential under the impact of salinity stress.

Source and disinfection of explants

Activity growing shoots of *Paulownia tomentosa* was collected as a source of explants from 7-year-old trees located at Sadat City. Explants were taken and washed in septal soap, then rinsed for 1 h with running tap water. Explant surface disinfection was performed aseptically using 70% ethanol for 1 min, followed by 25% (v/v) of Clorox (NaOCl 5.25%) for 5 min and then rinsed three times with sterilized distilled water. Thereafter, explants were immersed in 0.1% (w/v) of mercuric chloride ($HgCl_2$) for 1 min and rinsed twice with distilled sterilized water. Sterilized explants were used for the following experiments***:***

Shoot initiation

Various nutrient media, *i.e.,* Murashige and Skoog (MS) (1962), Woody Plant Medium (WPM) (Lloyd and McCown, 1980), and B5 (Gamborg's medium) Gamborg *et al*., 1968, were used. Shoot tips and stem segments were cultivated after surface sterilization on culture media augmented with benzyl amino purine (BAP) at 0.0, 0.1, 0.25, 0.5, and 1.0 mg/l to classify the most effective combination that has enhanced the highest proliferation. The percentage of survival, number of shoots, and shoot length were documented after 30 days in all treatments.

Shoot multiplication

For four weeks, primary shoots regenerated from explants were cultivated on the WPM basal medium supplemented with BAP and 2-isopentenyl-adenine (2iP) at 0.00, 0.50, 1.00, 2.00, and 3.00 mg/l, in combination with α-naphthaleneacetic acid (NAA) at 0.00, 0.25, 0.5, and 1.00 mg/l. Subsequently, the best cultures were separated and sub-cultured in the same media. After 30 days of culture, the mean number and mean length of shoots (cm) were noted.

NaCl effects on irradiated Paulownia shoot

Jars with small propagules of *Paulownia tomentosa* were irradiated in a gamma cell with Sc-137 with a dose rate of 0.723 rad/sec source at the Egyptian Atomic Energy Authority Radiation Technology Center, Nasr City, Cairo, Egypt. Shoots were irradiated with the doses of gamma rays at 0.0, 30, 60, 90, 120, and 150 Gy . The gamma-ray treated shoots were transferred from the jar and recultured on a fresh culture medium. Each treatment contains three replicates. After 30 days of culture, the survival percentages of shoots were recorded.

The individual shoots from irradiated cultures were grown on a hormone-free medium, supplemented with various concentrations of NaCl. The concentrations were 0.0, 1000, 2000, 3000, and 4000 mg/l of pure and dried NaCl. For NaCl tolerance, the irradiated shoots were screened *in vitro*. The vigorous shoots were selected and transferred to a fresh medium after six weeks of incubation and the survival rate in percentages were again recorded.

NaCl effects on NaN_3 treated Paulownia mutant shoot

Shoots derived from multiplication stage (3-5 cm long) were transferred to WPM medium (free hormones) containing different concentrations of sodium azide "NaN_3" at 0.0, 0.1, 0.2, 0.4, 0.8, and 1.0 mM for 5 min. On a new proliferation medium, the treated shoots with NaN_3 were transferred from the jar and recultured. Explants were cultivated in tissue culture jars in three replicates per treatment. After 30 days of culture, the survival percentages of shoots were logged.

For NaCl tolerance, the treated shoots were collected *in vitro*. The single shoot from treated cultures was grown on a hormone-free medium, supplemented with varying concentrations of NaCl. The concentrations were 0.0, 1000, 2000, 3000, and 4000 mg/l of pure and dried NaCl. The vigorous shoots were selected and transferred to a fresh medium after six weeks of incubation and the survival rate in percentages were again logged.

Root formation

Treated shoots at 2-3 cm were separated from multiplication cultures and inserted in the glass jars containing a rooting medium consisting of half-strength WPM macro and micronutrients, supplemented with indole-3-butyric acid (IBA) and NAA at 0.0, 1.0, 2.0, and 3.0 mg/l. The mean number of roots/shoots, mean length of roots, and mean length of shoots of *Paulownia tomentosa* during the rooting stage were recorded after 30 days.

Incubation conditions

In the growth chamber, cultures were incubated under controlled conditions. The temperature in the incubator was 25±2 °C, and a 'power' air conditioner controlled it. The photoperiod was automatically adjusted to 16 h of light and 8 h of darkness. Cool fluorescent bulbs supplied 3000 lux of lighting.

Inter simple sequence repeats (ISSR) technique

The CTAB technique (Bousquet *et al*., 1990) was used to extract DNA from 250 mg of fresh leaves generated from all treated plants (0, 30, 60, 90, 120, and 150 Gray; 0.00, 0.1, 0.2, 0.4, 0.8, and 1.0 mM NaN_3). According to the protocol identified and performed by Meyer *et al*. (1993), ISSR was conducted.

Acclimatization stage

The obtained plantlets (rooted shoots) were washed thoroughly with running tap water to discard media residues and treated with 0.2% (w/v) Moncut 25% (α,α,α-trifluoro-3′-isopropoxy-o-toluanilide) solution as a fungicide for 30 s and then, were transplanted *ex-vitro* in plastic pots (8 cm in diameter) containing soil potting mix of peat moss and sand (3:1). Pots were covered with transparent polyethylene bags and placed in a greenhouse. One week later, the covers were removed gradually, within one month. The percentage of survived transplants (%) was recorded. After one more month, the acclimatized plantlets were irrigated and fertilized every week to be ready for transplanting outside the greenhouse.

Statistical analysis

All the experiments had a completely random design. ANOVA was performed using the Co stat software program for statistical analysis. Duncan (1955) multiple range tests, as modified by Snedecor and Cochran (1990), were used to examine the significance of differences between the means of all treatments at the 5% level.

RESULTS

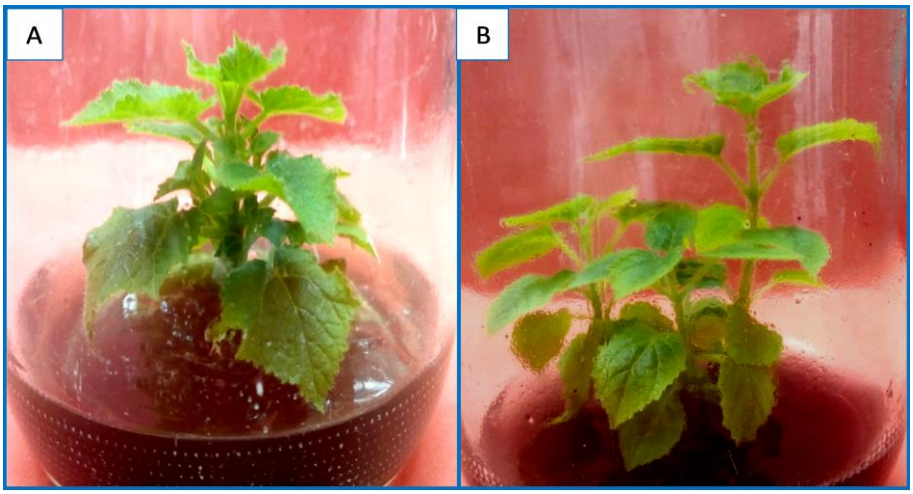
Shoot initiation

The effect of different explants and medium types with different concentrations of BAP on survival rate in percentage, the average number of shoots, and shoot length of *Paulownia tomentosa* is shown in Table 1 and Figure 1. As for WPM used with shoot tips and stem segments, the main effect of BAP exerted a very highly significant effect ($P \leq 0.05$) on the survival percentages at 80% and 87% survival, respectively, especially when the WPM culture medium was augmented with 1.0 mg/l BAP. Also, the same treatment achieved the highest value of shoots number, at 3.06 for shoot tips and 3.2 for stem segments, compared with the other treatments. Regarding shoot tips and stem segments, fortifying MS medium with BAP at 0.5 mg/l recorded the highest mean values of shoot length at 3.06 and 3.1 cm in series. The main effect of MS was that BAP exerted a highly significant effect ($P \leq 0.05$) on the given trait.

Table 1. Effect of medium types and different levels of BAP (mg/l) on survival rate (percentage), the average number of shoots/propagule and shoots, length of shoot tip and stem segment during the initiation stage of *Paulownia tomentosa* (Thumb).

Treatments		Shoot tips			Stem segments		
Medium type	BA conc.	Survival rate (%)	Average number of shoots/propagule	Shoots length (cm)	Survival rate (%)	Average number of shoots/propagule	Shoots length (cm)
MS	0.00	1.0^{k}	0.1^{h}	0.1^{i}	2.0^{m}	0.5^{i}	0.1^{h}
	0.10	50^{h}	0.3^{g}	2.5^{e}	55^{i}	0.7^{h}	2.6^{e}
	0.25	58^{f}	0.7^{e}	2.8^{c}	61^{f}	1.2^{f}	2.9^{c}
	0.50	65^{d}	1.06^{d}	3.06^{a}	70.66^{d}	1.5^{e}	3.1^{a}
	1.00	78^{b}	2.06^{b}	2.7^{d}	80.33^{b}	3.06^{b}	2.9^{c}
WPM	0.10	53^{g}	0.5^{f}	2.3^{g}	56^{h}	0.8^{g}	2.5^{f}
	0.25	60^{e}	1.06^{d}	2.7^{d}	65^{e}	1.7^{d}	2.8^{d}
	0.50	68^{c}	1.4^{c}	2.9^{b}	75^{c}	2.06^{c}	3.0^{b}
	1.00	80^{a}	3.06^{a}	2.5^{e}	87^{a}	3.2^{a}	2.6^{e}
B5	0.10	1.0^{k}	0.05^{h}	2.1^{h}	4.0^{l}	0.1^{k}	2.1^{g}
	0.25	20^{j}	0.1^{h}	2.4^{f}	25^{k}	0.4^{j}	2.5^{f}
	0.50	30^{i}	$0.\ 5^{f}$	2.7^{d}	35^{j}	0.8^{g}	2.8^{d}
	1.00	50^{h}	1.06^{d}	2.06^{h}	60^{g}	2.06^{c}	2.1^{g}

Means with different letters were significantly different at 5% level.



A) shoot tip. B) stem segment.

Figure 1. Initiation stage of *Paulownia tomentosa* using WPM medium containing 1.0 mg/l BAP.

Shoot multiplication

Data illustrated in Table 2 and Figure 2 exhibited the effect of WPM supplemented with various levels of BAP, 2iP, and different concentrations of NAA on the mean number of shoots and shoot length, resulting from shoot tips and stem segments. The interaction between 2iP and NAA both at 2.0 mg/l and at 0.5 mg/l expressed a highly significant effect ($P \leq 0.05$) on the trait under study, which achieved the highest mean value of shoots number (6 shoots/shoot tip and 7.5 shoots/stem segment). Moreover, the interaction between 2iP and NAA at 2.0 mg/l and at 0.25 mg/l, consecutively, recorded the highest significant value of shoot length (4.0 cm and 4.2 cm, respectively) compared with 0.1 cm for the control treatment. Increasing or decreasing the BA or 2iP concentration declined the mean number of shoots/propagula and shoot length.

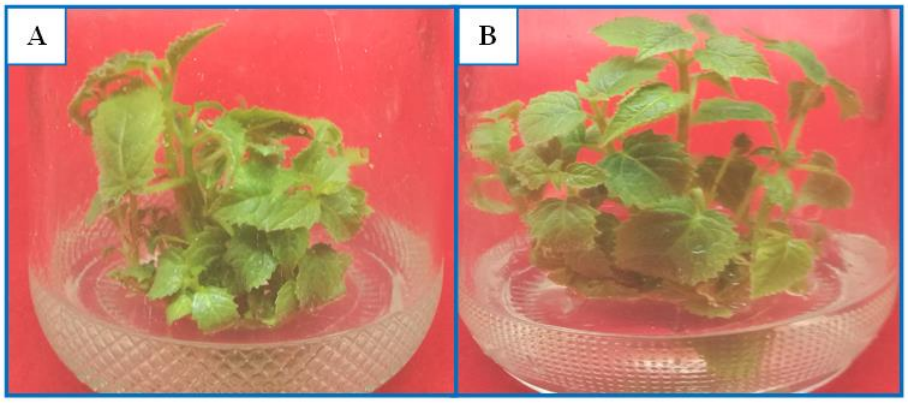


Figure 2. *Paulownia tomentosa* cultured on WPM medium supplemented. A) Without growth regulators (Control). B) With the combination of 2.0 mg/l BAP plus 0.50 mg/l NAA.

Table 2. Effect of WPM medium supplemented with BAP, 2iP (mg/l) and their combinations with NAA on average number of shoots/propagule and shoots length during multiplication stage of *Paulownia tomentosa* (Thumb).

Growth regulators conc. (mg/l)			Shoot tip		Stem segment	
BAP	2iP	NAA	Average number of shoots per propagule	Shoot length (cm)	Average number of shoots per propagule	Shoot length (cm)
0.00	0.00	0.00	0.10^{k}	0.1^{r}	1.0^{u}	0.1^{x}
0.00	0.00	0.25	0.10^{k}	0.1^{r}	1.2^{s}	0.2^{u}
0.00	0.00	0.50	0.40^{jk}	0.1^{r}	1.4^{q}	0.2^{u}
0.00	0.00	1.00	0.23^{jk}	0.1^{r}	1.3^{r}	0.2^{u}
0.5	0.00	0.00	0.33^{jk}	1.1^{p}	0.5^{v}	1.2^{u}
0.5	0.00	0.25	0.43^{jk}	1.1^{p}	2.0^{n}	1.2^{u}
0.5	0.00	0.50	0.4^{jk}	1.1^{p}	2.5^{l}	1.4^{t}
0.5	0.00	1.00	0.23^{jk}	1.1^{p}	2.3^{m}	2.16^{o}
1.00	0.00	0.00	0.93^{ijk}	2.1^{l}	1.0^{u}	2.5^{l}
1.00	0.00	0.25	2.33^{efg}	2.4^{k}	3.0^{i}	2.8^{j}
1.00	0.00	0.50	2.90^{def}	2.6^{i}	3.7^{g}	3.0^{h}
1.00	0.00	1.00	1.96^{gh}	2.5^{j}	3.5^{h}	3.2^{f}
2.00	0.00	0.00	0.56^{jk}	2.6^{i}	1.0^{u}	2.8^{j}
2.00	0.00	0.25	1.96^{gh}	2.7^{h}	1.1^{t}	3.2^{f}
2.00	0.00	0.50	4.33^{b}	2.0^{m}	6.0^{b}	2.3^{m}
2.00	0.00	1.00	3.16^{cde}	1.5^{o}	5.0^{c}	2.1^{p}
3.00	0.00	0.00	1.60^{ghi}	3.0^{e}	2.0^{n}	3.6^{d}
3.00	0.00	0.25	1.60^{ghi}	3.5^{c}	1.3^{r}	3.2^{f}
3.00	0.00	0.50	1.16^{hij}	2.9^{f}	1.4^{q}	2.3^{m}
3.00	0.00	1.00	0.90^{ijk}	2.0^{m}	1.0^{u}	2.2^{n}
0.00	0.50	0.00	0.56^{jk}	1.06^{q}	1.5^{p}	1.0^{v}
0.00	0.50	0.25	0.80^{ijk}	1.1^{p}	2.7^{k}	1.0^{v}
0.00	0.50	0.50	0.93^{ijk}	1.7^{n}	3.0^{i}	1.8^{r}
0.00	0.50	1.00	0.90^{ijk}	1.5^{o}	2.8^{j}	1.6^{s}
0.00	1.00	0.00	1.56^{ghi}	2.0^{m}	1.6^{o}	2.0^{q}
0.00	1.00	0.25	2.90^{def}	3.1^{d}	3.8^{f}	3.0^{h}
0.00	1.00	0.50	3.46^{bcd}	3.0^{e}	4.5^{d}	2.8^{j}
0.00	1.00	1.00	3.00^{de}	2.5^{j}	4.0^{e}	2.53^{k}
0.00	2.00	0.00	0.70^{ijk}	1.1^{p}	1.4^{q}	2.0^{q}
0.00	2.00	0.25	1.50^{ghi}	4.0^{a}	2.0^{n}	4.2^{a}
0.00	2.00	0.50	6.00^{a}	3.5^{c}	7.5^{a}	3.7^{c}
0.00	2.00	1.00	4.00^{bc}	3.0^{e}	6.0^{b}	3.1^{g}
0.00	3.00	0.00	2.00^{fgh}	3.1^{d}	2.3^{m}	3.6^{d}
0.00	3.00	0.25	1.50^{ghi}	3.6^{b}	2.0^{n}	3.8^{b}
0.00	3.00	0.50	$1.00^{i.jk}$	3.0^{e}	1.4^{q}	3.4^{e}
0.00	3.00	1.00	0.9^{ijk}	2.8^{g}	1.2^{s}	2.9^{i}

Means with different letters were significantly different at 5% level.

NaCl effects on irradiated Paulownia shoot

As for the survival rates of cultured shoots exposed to various levels of NaCl (mg/l) and gamma-ray doses (Table 3), the main effect of NaCl levels produced very highly significant effect ($P \leq 0.05$) on the given trait. Regarding the NaCl level, data clearly showed that the highest significant value of percentage of survival was noticed with control treatment (100%). Increasing NaCl levels significantly decreased survival.

Regarding gamma-ray dose irrespective of NaCl level, data showed that shoots exposed to 60 Gy showed good growth and achieved the highest survival rate (95.32%), as shown in (Figure 3). On the contrary, shoots exposed to higher doses gave the lowest significant percent rate. Interaction between NaCl and gamma-ray showed that shoots irradiated with 60 Gy and cultured on 0, 1000, 2000, 3000, and 4000 ppm NaCl could survive with a higher percentage rate (100, 100, 100, 100, and 76.61 respectively). On the other hand, shoots exposed to 150 Gy and cultured on NaCl at different levels showed the lowest significant rate of percentages.

Table 3. Effect of NaCl concentration (mg/l) on survival rate in percentages of Paulownia shoots irradiated at different gamma doses.

NaCl mg/l	Gamma doses (Gray)						Mean NaCl	Significance		
	Control	30	60	90	120	150		Doses	NaCl	Does X NaCl
Control	100^{a}	100^{a}	100^{a}	43.3^{c}	13.3^{ef}	1.0^{g}	72.3^{a}			
1000	80^{b}	80^{b}	100^{a}	26.6^{d}	11.6^{efg}	1.0^{g}	60.6^{b}			
2000	1.0^{g}	1.0^{g}	100^{a}	23.3^{de}	5.0^{fg}	1.0^{g}	27.0^{c}	***	***	***
3000	1.0^{g}	1.0^{g}	100^{a}	13.3^{ef}	1.0^{g}	1.0^{g}	24.2^{d}			
4000	1.0^{g}	1.0^{g}	76.6^{b}	10^{fg}	1.0^{g}	1.0^{g}	18.9^{e}			
Mean of doses	36.6^{b}	36.6^{b}	95.33^{a}	23.3^{c}	6.4^{d}	1.0^{e}				

Means with different letters were significantly different at 5% level.

Figure 3. *Paulownia tomentosa* shoots treated by 60 Gy showed good growth and higher survival rate irrespective of NaCl level.

NaCl effects on NaN_3 treated Paulownia mutant shoot

The effect of NaCl on the survival of *Paulownia tomentosa* mutant shoots in percentage, is shown in Table 4 and Figure 4. The data indicated that the main effect of NaCl has a very highly significant effect ($P \leq 0.05$) and expressed an inverse relationship between the shoots and the NaCl treatments, where at control treatment, shoot survival resulted with the highest percentage at 95.0%, then declined to 7.5% at the highest level of NaCl treatment (4000 mg/l). Likewise, NaN_3 concentrations also produced a very highly significant effect ($P \leq 0.05$) on the given trait. As the NaN_3 concentration increased up to 0.8 mM, the survival rate in percentage increased to 72.66%, then decreased to 19.06% at 1.0 mM of NaN_3. The interaction between both variables exerted a significant effect ($P \leq 0.05$) on the studied character, and the highest survival rate (100%) were noticed with NaCl control treatment and the NaN_3 treatments at 0, 0.1, 0.2, 0.4, and 0.8 mM.

In general, from the results in Tables 3 and 4, it could be concluded that the above-mentioned results revealed that using gamma rays is recommended for maximizing the survival of *Paulownia tomentosa* mutant shoots on different levels of NaCl compared with NaN_3 treatments.

Table 4. Effect of NaCl concentration (mg/l) on survival rate in percentages of Paulownia shoots treated with different NaN_3 concentrations.

NaCl mg/l	NaN_3 concentration (mM)						Mean NaCl	Significance		
	0.0	0.1	0.2	0.4	0.8	1.0		NaN_3	NaCl	NaN_3 X NaCl
Control	100^{a}	100^{a}	100^{a}	100^{a}	96.6^{ab}	73.3^{de}	95^{a}			
1000	80^{cde}	80^{cde}	83.33^{cd}	60^{f}	86.6^{bc}	10^{h}	66.6^{b}			
2000	1.0^{h}	33.3^{g}	26.66^{g}	13.3^{h}	76.6^{cde}	10^{h}	26.8^{c}	***	***	****
3000	1.0^{h}	10^{h}	13.33^{h}	8.3^{h}	70^{ef}	1.0^{h}	17.2^{d}			
4000	1.0^{h}	5.0^{h}	3.66^{h}	1.0^{h}	33.3^{g}	1.0^{h}	7.5^{e}			
Mean NaN_3	36.6^{c}	45.6^{b}	45.4^{b}	36.5^{c}	72.6^{a}	19.0^{d}				

Means with different letters were significantly different at 5% level.

Figure 4. *Paulownia tomentosa* shoots treated with 0.8 mM NaN_3 and a control medium without NaCl.

Root formation

The recent results further disclosed the effect of half-strength WPM medium and both IBA and NAA (mg/l) on the mean number of roots/shoot, mean length of roots, and mean length of shoots of *Paulownia tomentosa* during the rooting stage (Table 5 and Figure 5). In terms of augmenting the WPM medium with various levels of IBA and NAA showed a very highly significant ($P \leq 0.05$) effect on the given trait. The greatest number of roots/shoot (6.0) was formed on half strength of the WPM medium with NAA at 2.0 mg/l, while the longest root (9.0 cm) and shoot (8.33 cm) were obtained on half strength of the WPM medium with IBA at 1.0 mg/l. In contrast, the lowest number of roots/shoot (1.66) was produced using half strength of the WPM with 3.0 mg/l IBA. The lowest root length was achieved on half strength of the WPM with 1.0 mg/l NAA. In general, increasing IBA and NAA concentration decreased shoot length and roots number.

Table 5. Effect of WPM medium supplemented with IBA and NAA on average roots/explant, average shoot length, and average root length during rooting stage of *Paulownia tomentosa* (Thumb).

Auxin conc. (mg/l)		Average roots/explant	Average shoot length (cm)	Average root length (cm)
IBA	NAA			
0.00	0.00	0.1^{e}	0.1^{g}	7.33^{b}
1.00	0.00	4.66^{b}	9.0^{a}	8.33^{a}
2.00	0.00	3.33^{c}	7.0^{b}	6.66^{bcd}
3.00	0.00	1.66^{d}	4.3^{c}	6.0^{d}
0.00	1.00	4.33^{bc}	2.5^{f}	7.0^{bc}
0.00	2.00	6.0^{a}	3.0^{d}	6.33^{cd}
0.00	3.00	4.0^{bc}	2.7^{e}	6.0^{d}

Means with different letters were significantly different at 5% level.

Figure 5. Rooting stage of Paulownia using half-strength WPM medium. A) Without growth regulators (Control), B) Medium containing 1.0 mg/l IBA.

Acclimatization stage

Acclimatization of mutant *Paulownia tomentosa* plantlets was done for 10 weeks (Figure 6). In general, increasing gamma doses or the NaN_3 concentrations decreased survival rate. Therefore, we found that the rate of survival in percentage decreased from 90% to 10%.

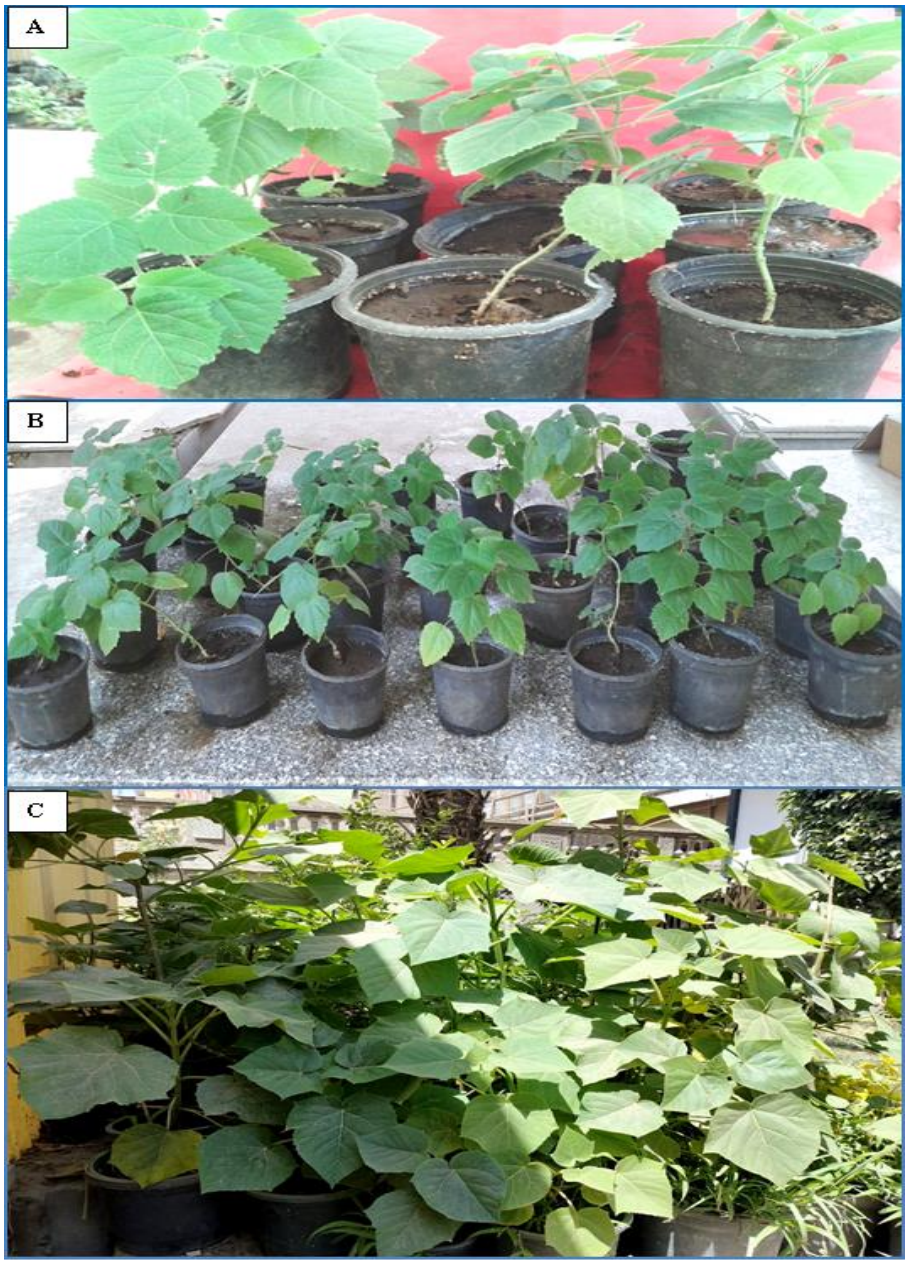


Figure 6. Acclimatization stage of Paulownia derived tissue culture-mutant plantlets. A) After 10 weeks. B) After 20 weeks. C) After 45 weeks.

Inter simple sequence repeats (ISSR) technique

A total of nine ISSR primers were used to analyze *Paulownia tomentosa* mutant plantlets caused by gamma rays and NaN_3. ISSR primers resulted in a total generation of 50 reproducible ISSR-bands: 29 were monomorphic and 21 were polymorphic. The gross percentage of polymorphism was 42.85% (Table 6 and Figure 7). With the 17899B and 17898B primers, the maximum number of polymorphic bands (four) was obtained. However, with two primers, including the UBC867 and UBC828, the minimum number (one) was obtained. For primer 17898B, the polymorphism percentage varied from 16.66% (primer UBC867) to as high as 80 %.

Table 6. The total number of bands for mutant plant (gamma ray and NaN_3), polymorphism %, monomorphic % and unique bands of ISSR markers obtained by nine random primers.

Primers	Total band	Polymorphic		Monomorphic		Unique band		Band size
		Number	%	Number	%	Number	Molecular weight	
UBC824	3	2	66.66	1	33.33	1-	1000	750-1000
UBC828	5	1	20	4	80	1+	1000	500-1000
UBC826	4	2	50	2	50	-	-	500-1000
UBC862	5	2	40	3	60	-	-	200-750
UBC873	6	2	33.33	4	66.66	1-	750	500-1000
UBC867	6	1	16.66	5	83.33	1-	1000	200-1000
17898B	5	4	80	1	20	1+	1000	500-1000
17898A	7	3	42.85	4	57.14	-	-	100-750
17899B	9	4	44.44	5	55.55	-	-	100-750
Total	50	21	42.85	29	56.22			

1- refers to the absence of the unique band; 1+ refers to the presence of the unique band.

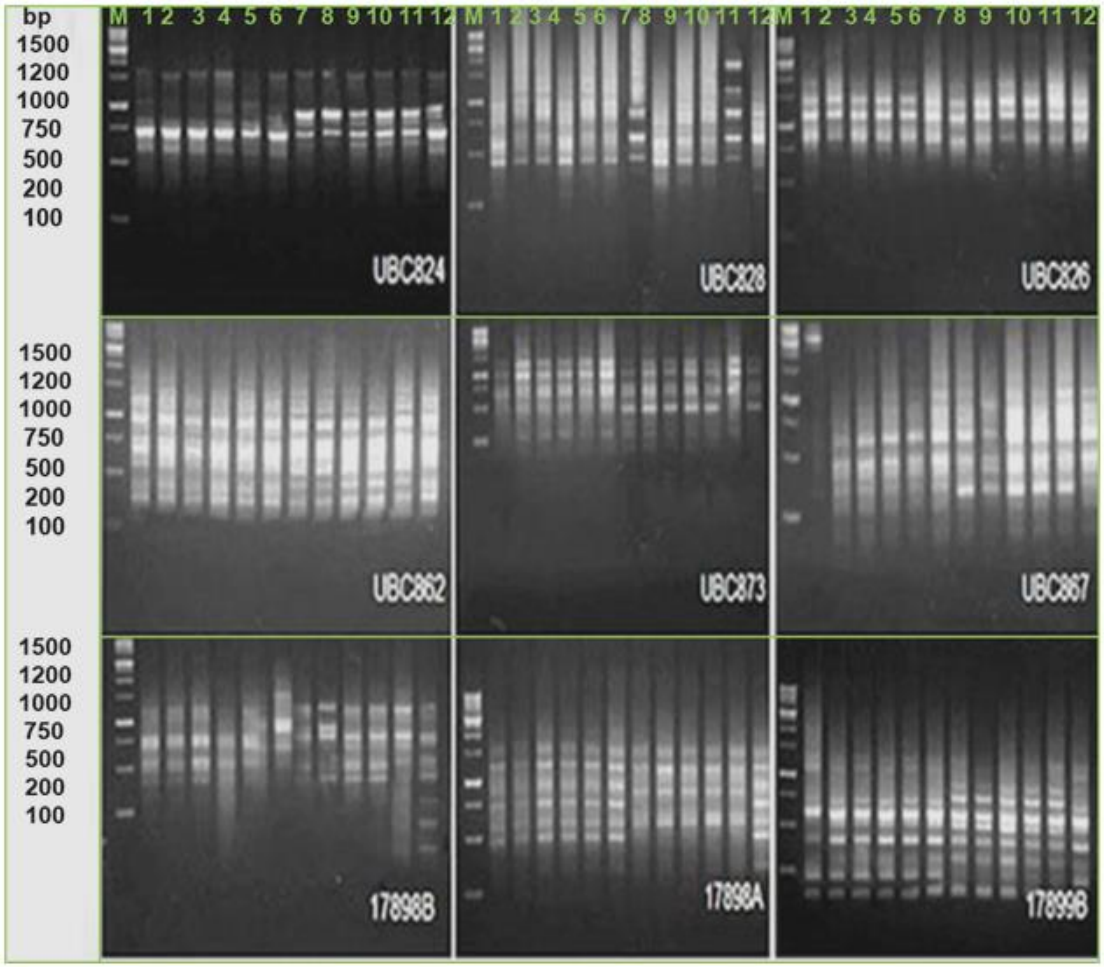


Figure 7. ISSR analysis for *Paulownia tomentosa* shoots showed genetic polymorphism, monomorphic, and unique as a result of different doses of gamma ray and different concentrations of NaN_3. M = Marker 1 = 0 Gray, 2 = 30 Gray, 3 = 60 Gray, 4 = 90 Gray, 5 = 120 Gray, 6 = 150 Gray, 7 = 0.0 mM NaCl, 8 = 0.1 mM NaCl, 9 = 0.2 mM NaCl, 10 = 0.4 mM NaCl, 11 = 0.8 mM NaCl, and 12 = 1.0 mM NaCl.

At 120 Gy of gamma-ray, one fragment with primer UBC824 vanished and one fragment with primer 17898B at 150 Gy appeared. In comparison, one fragment with primers of either UBC873 or UBC867 at 1.0 mM and 0.8 mM of NaN_3, respectively, and one fragment with primer UBC828 at 0.8 mM of NaN_3 appeared, which can be considered a positive marker of Paulownia salt tolerance.

DISCUSSION

The growth media's composition greatly influences plant tissue growth and morphogenesis. Plant tissue culture media contain macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, carbon sources, stabilizing agents, and growth regulators. MS medium has a lot of nitrate, potassium, and ammonium. Thus, it is good for plant growth in general (Pierik, 1987).

Results showed that the maximum significant values of shoot tip and stem segments survival rate in percentages (80% and 87%, respectively), as well as, shoot numbers were obtained on the WPM medium augmenting with 1.0 mg/l BA. In this respect, the WPM, which has a low total ion content but a high sulphate and magnesium concentration to encourage the growth of woody plant tissue, is a medium specifically designed for perennial or woody plants (Nas *et al*., 2013). Half MS medium contained 20 g/l sucrose and 0.5 mg/l BAP is thought to be the most effective for micro-clonal proliferation, whereas 0.2 mg/l BAP causes plants to grow slowly (Pożoga *et al*., 2019).

Plant growth regulators include cytokinins, auxins, gibberellins, ethylene, and abscisic acid, among others. The interaction and balance between the growth regulators given in the medium and the growth chemicals produced endogenously regulate growth and morphogenesis *in vitro* (George, 1993). Cytokinins increase the division of plant cells and have been linked to various physiological and developmental processes. These impacts include delaying senescence in detached organs, nutrition mobilization, chloroplast maturation, and morphogenesis regulation (Taiz and Zeiger, 1991).

In our investigation, the interaction between 2iP and NAA at both 2.0 mg/l and 0.5 mg/l. respectively, expressed a highly significant effect ($P \leq 0.05$) on the trait under study. which achieved the highest mean value of shoots number (6 shoots/shoot tips and 7.5 shoots/stem segments). Similar findings were recorded by Fahmy and Gendy (2018) on Paulownia hybrid (*P. elongata* × *P. fortunei*) and Abdi *et al*. (2013) on *Aloe vera*, and Krishnan *et al*. (2018) on *Ophiorrhiza mungos*. This finding could have taken place due to the accurate balance between both exogenous growth regulators and those of endogenous biosynthesis hormones, which resulted in the best gene expression and subsequently the growth and development.

In vitro-induced mutagenesis successfully creates genetic variation, selection, and replication of mutant clones (Penna *et al*., 2012). In terms of biological effects induced by a counterintuitive switch from low-dose stimulation to high-dose inhibition, gamma irradiation has been widely applied in medicine and biology (Charbaji and Nabulsi, 1999). Previous research has shown that low-dose ionizing irradiation on plants and photosynthetic microorganisms results in increased cell proliferation, cell growth, enzyme activity, stress resistance, and crop yields (Chakravarty and Sen, 2001).

The *vitro* mutagenesis is a combination of *in vitro* culture and mutation induction that increases variability in economically important cultivars or is used on plants in developing agriculturally productive varieties (Jain *et al*., 1998, Abdrabou *et al*., 2017; Abbas *et al*., 2021; Al-Taweel *et al*., 2021; Azzam *et al*., 2021). The technique of induced mutation is a valuable tool that has not yet been fully utilized in fruit breeding (Predieri and Gatti, 2003). Tissue culture improves efficiency by allowing for the handling of large populations, as well as, increasing mutation induction efficiency, mutant recovery potential, and the speed with which selected variants can be cloned (Azzam and Khalifa, 2016; Predieri and Gatti, 2000).

In our study, the survival rate in the control treatment (0.0 Gy) was extremely low. Nevertheless, gamma rays at 60 Gy achieved the highest percentage at 95.33%. Increasing Gy dose above 60 Gy was concomitant with a clear and progressive decrease of survival rate as shown in the cases of both 90 Gy and 120 Gy. As the NaN_3 concentration increased up to 0.8 mM, the percent of survival rate increased to 72.66%, then decreased to 19.06% at 1.0 mM of the NaN_3.

Increasing the NaCl level reduced the survival rate significantly when compared with the control. Salinity-induced growth enhancement has been reported for a variety of *in vitro* cultures, including *Suaeda aegyptiaca* (Eshel, 1985), *Prunus cerasifera*

peach rootstock (Dimassi-Theriou, 1998), and date palm (Al-Khayri, 2002). Furthermore, a lower seawater level caused shoot multiplication in two jojoba clones (Fayek *et al*., 2010). The increased osmolarity of NaCl may explain its beneficial effect on plant growth (Flowers and Lauchli, 1983).

According to our findings, the most roots/shoot (6.0) was formed on half strength of the WPM medium strength with NAA at 2.0 mg/l, while the longest root (9.0 cm) and shoot (8.33 cm) were obtained on the WPM medium at half strength with IBA at 1.0 mg/l. The formation of roots was stimulated by high levels of auxin relative to cytokinin. In this respect, Roy (2015) stated that Paulownia shoots rooted well in half-strength MS with 2.0 mg/l NAA. Hassan *et al*. (2018) reported that both IBA and NAA at 0.5 or 1 mg/l gave the highest significant root number/shoot. Meanwhile, auxin at 4 mg/l gave the highest significant root lengths. Ultimately, Mohamad *et al*. (2021) recorded that adding 1.0–1.5 mg/l of IBA or NAA to the medium significantly enhanced the number of roots/plantlets and the longest root length. Finally, Mohameed and Alkhalifa (2022) showed that IBA at a concentration of 1 mg/l resulted in the formation of roots with the highest average of 12 roots and a length of 6.6 cm.

Acclimatization of *in vitro* plantlets was affected by several factors, such as, acclimatization media (Mishra *et al*., 2011), light intensity, and moisture (Hazarika, 2003). According to our research, increasing gamma doses or NaN_3 concentrations reduced survival rate. As a result, the survival rate dropped from 90% to 10%. Peat-soil mixtures were effective for acclimatizing Paulownia rooted shoots (Bahri and Bettaieb, 2013; Zayova *et al*., 2014). Taha and Seleem (2021) showed that more than 90% of the acclimatized plants successfully survived.

Traditional methodologies for mutant plant choice based on morphological and biochemical signs are much less reproducible because they affect environmental factors. Mutation detection using PCR and non-PCR tactics is consequently extra dependable and repeatable and has been used to display numerous crop mutants. The maximum honest utility of PCR for mutation evaluation is to discover the presence or absence of complex DNA sequences (Khan *et al*., 2009; Azzam *et al*., 2022).

In our research, gamma rays at 120 and 150 Gray and 1.0 mM and 0.8 mM of NaN3 improved genetic variability. Other researchers reported that a gamma-ray dose of 0.1 kGy improved genetic variability but that a dose of 0.2 kGy increased differences in plant growth and production, as well as, molecular heterogeneity in soybeans and peanuts (Ferguson *et al*., 2004; Hanafiah *et al*., 2010). These gamma-ray effects have been linked to DNA structural rearrangements (breaks, transpositions, deletions, and so on) resulting from various types of DNA disruption (Silva *et al*., 2010). Miri *et al*. (2009) utilized RAPD markers to identify genetic differences between induced banana mutant clones (*Musa acuminata* cv.).

CONCLUSIONS

In conclusion, this study identified an *in vitro* propagation protocol of *Paulownia tomentosa* through shoot tip and nodal stem segments. The WPM medium, supplemented with 1.0 mg/l BAP, was the best initiation medium. The addition of 2ip at 2.0 mg/l to the WPM appeared to be the most effective treatment at the multiplication stage. The highest rooting percentage (100%) was achieved by adding 2.0 mg/l NAA to the WPM medium at half strength. The obtained results showed that the overlapping between 0.00 level of NaCl and Gy dose up to 60 Gy recorded the highest survival percentage rate (100%). Meanwhile, the interaction between NaCl levels (control and 3000 mg/l) with 60 Gy brought about the highest percentage of survival (100%). Increasing gamma doses or NaN_3 concentrations reduced survival rates. As a result, the survival rate dropped from 90% to 10%. At 120 Gy of gamma-ray, one fragment with primer UBC824 vanished and one fragment with primer 17898B at 150 Gy appeared. In comparison, one fragment with primer, either UBC873 or UBC867 at 1.0 mM and 0.8 mM of NaN_3, respectively, and one fragment with primer UBC828 at 0.8 mM of NaN_3 appeared, which can be considered as a positive marker of Paulownia salt tolerance.

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