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Effect of different concentrations and combinations of some plant growth regulators on *Punica granatum* anther culture

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A B S T R A C T

Plant growth regulators (PGR), also known as plant hormones, are a variety of chemical compounds that have a significant impact on the development and differentiation of plant cells. This work aimed to study the effect of various combinations of Plant Growth Regulators (PGRs) for example [6-Benzyladenine (BA), 2,4-dichlorophenoxyaceticg acid (2,4-D), Naphthalene acetic acid (NAA) and indol-3-ylbutyric acid (IBA)] on callus induction and shoot regeneration from anther culture of S1 progenies resulted from Manfalouty cultivar open pollinated. The results showed that highest value of percentage of callus induction was 10.6% on MS medium supplemented with 1 mg / l of NAA and 2 mg / l of BA also highest value of percentage of shoot formation 60 % on MS medium supplemented with 3 mg/ l of NAA and 2 mg /l of BA.

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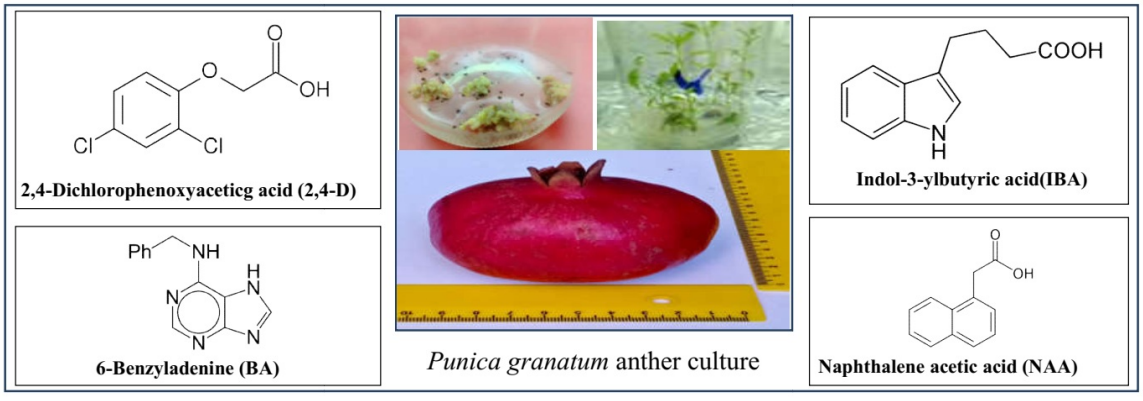

Graphical Abstract

1. Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits. It is a favorite table fruit of the tropical and subtropical regions in the world. It is grown in every humid tropical region. Pomegranate (*Punica granatum* L.) a member of family Punicaceae, which has only one genus (Punica) and two species P. granatum and P. protopunica, with

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chromosomal numbers of 2n = 16 and 18, respectively.[1-2] In Egypt, the total cultivated area of pomegranate is about 80,000 fed and produced about 400,000 tons. About 100,000 tons are exported annually.

Pomegranate is propagated vegetatively through layering and hard wood cuttings. Micropropagation in fruit trees could aid in overcoming the difficulties of vegetative propagation, resulting in true-to-type plants, as well as rapid and large production of disease-free planting materials.[1] As a result, various studies on pomegranate tree micropropagation have been done in recent years. *Punica granatum* L. regeneration protocols have been devised include. Protocols for regeneration of pomegranate through either organogenesis from callus derived from leaf segments,[3-7] cotyledons,[8] nodal segment,[9] mature leaf explants,[10] anthers,[11-12] or through embryogenesis from various seedling explants, petals and immature zygotic embryos.[5]

Doubled haploids have played an essential role in the production and use of structured mutant populations for forward and reverse genetics, as well as in basic and practical genetic investigations of crop plants.[13] DHs have become important tools for genome mapping, providing reliable information on the location of major genes and QTLs for economically important traits.[14]

Since the 1970s, a lot of work has gone into producing haploids for fruit tree breeding using gametic embryogenesis, although it hasn't always worked successfully.[15] In a variety of economically important crop species, including major cereals and Brassicas, doubled haploid techniques such as anther and microspore cultures, broad hybridisation, ovary and ovule cultures have been well established.[16] Many studies have been conducted to improve the anther culture response in pomegranate species by using different combinations of plant growth regulators.[11-12] This work aimed to study of the effect of four plant growth regulators, namely, [6-Benzyladenine (BA), 2,4-dichlorophenoxyaceticg acid (2,4-D), Naphthalene acetic acid (NAA) and indol-3-ylbutyric acid (IBA)] (**Fig. 1**), for establishment protocol for callus induction and shoot formation of pomegranate through anther culture. These chemical compounds were used before in different cases, for example, the effectiveness of 6-benzyladenine on flowering of Arabidopsis thaliana was studied before, and also the effect of indol-3-ylbutyric acid on alkaloid content in *Nicotiana rustica* stem cuttings was reported before.[17-18]

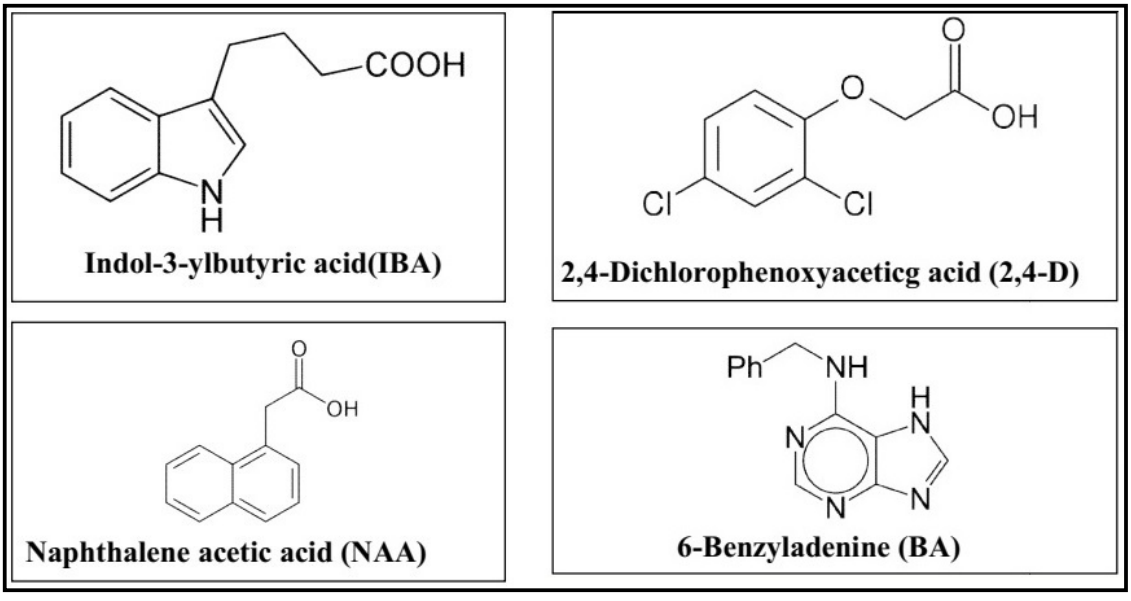

Fig. 1. The chemical structure of the four used plant growth regulators.

2. Results and Discussion

2.1 Effect of different NaOCl concentrations on anther of (Punica granatum L) cultured in vitro:

Data in **Table 1** show the effect of surface sterilization with NaOCl at different concentrations (20, 30, and 40%) for 20 minutes on survival and contamination percentage of anther explants of *Punica granatum* after 4 weeks in culture .

Data show a significant difference in the survival percentage of explants. The highest percentage of survival was 73 %, which was achieved by using NaOCl at 30%. On the other hand, the lowest percentage of survival was 32 and 40%, which resulted by using NaOCl at 40% and 20% respectively.

Data show a significant difference in the percentage of explant contamination. The highest percentage of contamination was 12%, which was achieved by using NaOCl at 20%. On the other hand, the lowest percentage of contamination was 2% and 7%, which resulted by using NaOCl at 40% and 30% respectively.

Table 1. Effect of surface sterilization by using different concentrations of NaOCl on survival and contamination percentage of *Punica granatum* anther explants.

Clorox concentrations	Survival (%)	Contamination (%)	Mortality (%)
20%	40[b]	12[a]	22[b]
30%	73[a]	7[c]	30[b]
40%	32[a]	2[c]	14[a]

2.2 Effect of different concentration of growth regulators on initiation of callus from anther culture:

Anther of pomegranate were cultured on MS media with different concentration of growth regulators at **Table 2**. After 4 week of inoculation the Growth parameters and morphological characterize are represented in **Table 2** and **Fig. 2.** Data in **Table 2** and **Fig. 2** showed that the survival of anther was affected by increasing 2.4.D level in medium. The highest mean (26.8 %) obtained on medium supplemented with 1 mg l of 2.4.D, while the increasing concentration of 2.4.D lead to reduced survival rate.

Increasing the concentration of 2.4.D in medium to 5 mg /l resulted in the lowest average of callus survival with (9.8%). When BA and NAA used the best medium for survival rate of anther MS medium supplemented with 1 mg l of NAA and 2 mg l of BA that recorded the highest mean of survival rate with (22.6%).

Data in **Table 2** and **Fig. 2** showed that there were significant differences of callus induction percentage. From this data, the highest value of percentage of callus induction was 10.6%. on MS medium supplemented with 1 mg l of NAA and 2 mg l of BA, while the lowest value of percentage of callus induction was 4% and was recorded on MS medium supplemented with 5 mg l of 2.4.D

In addition, data in **Table 2** and **Fig. 2** showed that Brown anther per explant was affected by different concentrations of 2.4.D in medium. The lowest mean (73.20) obtained on medium contains 1 mg/L of 2.4.D, while the increasing concentration of 2.4.D lead to increasing the brown anther. Increasing the concentration of 2.4.D in medium to 5 mg/l resulted in the highest average of brown anther (90.2).

These results are in agreement with the findings reported before[19] that indicated anthers of pomegranate were incubated on MS nutrient medium supplemented with BA and NAA. Callus from anther walls were recorded after 30 days of culture, subsequently adventitious shoots were formed by cultured callus to a medium treated with 2 µM of BA and 0.5 µM NAA. Regenerated shoots had a diploid chromosome number of 16. Also, it was reported that culturing of anthers of Citrus aurantifolia with uninucleate pollen grains at the tetrad stage on modified Murashige and Skoog medium supplemented with 0.5 mg /l BA and 1 mg / l NAA for 20-30 days differentiated into embryoids.[20]

Table 2. Effect of using different concentrations of growth regulator on initiation of Callus of pomegranate through anther explants.

Medium code	growth regulator mg/l			Survival (%)	Brown anther	Callus formation (%)
	2.4.D	BA	NAA			
1	1	-	-	26.8	73.2	7
2	3	-	-	21.6	78.4	5.8
3	5	-	-	9.8	90.2	4
4	-	2	2	13.5	86.5	7
5	-	2	1	20.4	79.6	10.6
6	-	4	0.5	22.6	77.4	9.8

2.3. Effect of different combinations between BA and NAA on callus differentiation:

Data in **Table 3** and **Fig. 2** show the effect of different combinations between BA at 1.0, 2.0 and 3.0 mg/l and different concentrations of NAA at 1.0, 2.0 and 3.0 mg/l on callus differentiation of *Punica granatum* anther culture, as shown in **Fig. 2**.

The data clearly demonstrate that there was a significant difference between different combinations of BA and NAA with shoot formation percentage of callus. By increasing the concentrations of BA to 3.0 mg/l with NAA at 3.0 mg/l, the shoot formation percentage to 60 %. However, using the treatment BA to 1.0 mg/l + NAA at 3.0 mg/l resulted in the lowest percentage of shoot formation 10 %.

There was a significant difference between different combinations of BA and NAA with shooting numbers. Medium containing BA to 3.0 mg/l + NAA at 3.0 mg/l recorded the highest number of shoots 4.1 shoot/explant while the medium containing BA at 1.0 mg/l + NAA at 3.0 mg/l recorded the lowest number of shoots 0.9 shoot/explant. There was no significant difference between different combinations of BA and NAA with shooting length. The highest shoot length 2.2 cm was recorded with 3.0 mg/l BA + 3.0 mg/l NAA while, the lowest results 1.2 cm, resulted in BA 1.0 mg/l + NAA at 1.0 mg/l.

Table 3. Effect of different combinations between BA and NAA on callus differentiation of *Punica granatum* anther explant

Treatments	Shoot formation (%)	Shoot number	Shoot length(cm)
1.0 BA + 1.0 NAA	20.0^{bc}	3.1^{ac}	1.2^{a}
1.0 BA + 2.0 NAA	13.5^{c}	1.9^{ad}	1.3^{a}
1.0 BA + 3.0 NAA	10.0^{c}	0.9^{cd}	1.5^{a}
2.0 BA + 1.0 NAA	23.0^{bc}	3.3^{ab}	1.3^{a}
2.0 BA + 2.0 NAA	30.0^{b}	2.0^{ad}	1.3^{a}
2.0 BA + 3.0 NAA	19·0^{bc}	1.5^{bd}	1.6^{a}
3.0 BA + 1.0 NAA	19.0^{bc}	2.0^{ac}	1.6^{a}
3.0 BA + 2.0 NAA	22.5^{bc}	2.2^{ac}	1.9^{a}
3.0 BA + 3.0 NAA	60.0^{a}	4.1^{a}	2.2^{a}

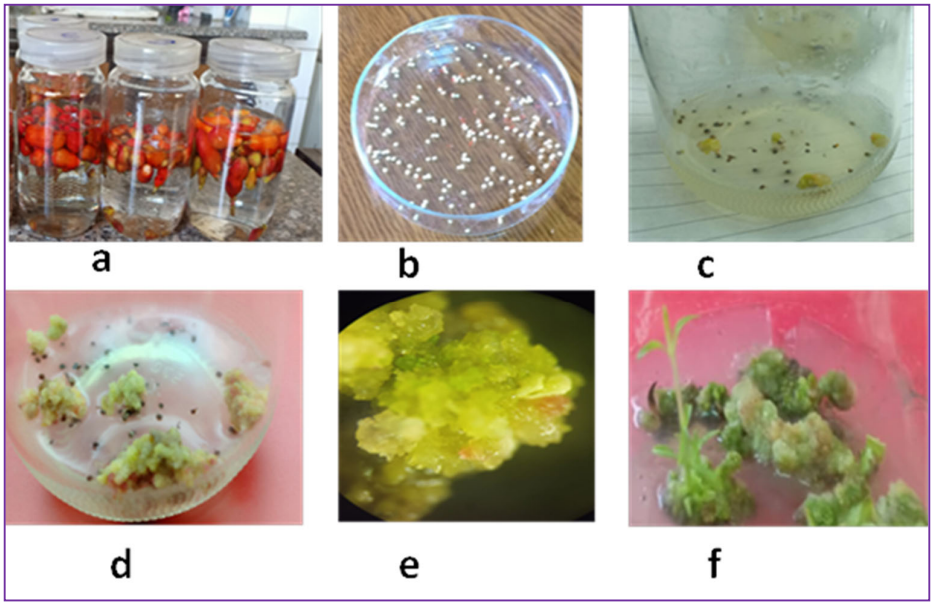

Fig. 2. callus induction and shoot formation of pomegranate through anther culture (a)Sterilization of explants (b) Sterilized anther (c) Callus formation on anther after 30(d) Callus formation on anther after 60 days proliferation stage (e) Embrogenic callus (f) Shoot formation from callus.

2.4 Effect of different concentrations of IBA on roots induction:

In this experiment, shoots were cultured on MS basal medium supplemented with different concentrations of IBA at 0.0, 0.5, 0.,75 and 1.0 mg/l. As shown in **Table 4** and **Fig. 3** roots number was affected significantly by concentrations of IBA. The best concentration of IBA to improve growth and root formation was 0.75 mg/l IBA where root formation number was 7.0 root/shoot. Increasing a concentration of IBA over that to 1.0 mg/l reduces the number of roots 4.0 root/shoot. The low concentration of IBA at 0.5 mg/l recorded the lowest roots number 2.0 root/shoot. IBA at 0.0 mg/l does not reduce any roots.

There was no significant difference of root length due to different concentrations of IBA. Using IBA at 0.75 mg/l recorded root length 4.5 cm rather than using IBA at 0.5 mg/l which resulted in 3.0 cm for both treatments, as shown in **Fig. 3**.

These results were in agreement with the data reported before.[21] In all pomegranate genotypes, from four commercial pomegranate genotypes cultivated in Egypt (Manfalouty, Tahrir, Badr, and Araby). Adding 1.0 mg/l IBA to MS basal medium enhanced shoot formation, roots ratio (100 percent), and plantlet growth. The genotype Manfalouty had the highest estimated regeneration frequency of 79.75 percent, followed by 70 percent, 63 percent, and 55 percent in Tahrir, Badr, and Araby, respectively. Some reported papers stated that addition of an auxin to the medium was essential to induce rooting in the regenerated shoots.[22] Root initiation occurred within 10–15 days on half-strength MS medium supplemented with 0.054–5.4 mM NAA. However, the greatest percentage of shoots was rooted on a medium containing 0.54 mM NAA.[23] mentioned that Best average rooting of pomegranate response was observed on MS medium treated with 0.50 mg/l NAA (97%) and 0.50 mg/l IBA (95%). As a result, both NAA and IBA revealed the same rooting response. Finally, we can conclude from our result that the highest value of callus induction was on MS medium supplemented with 1 mg l of NAA and 2 mg l of BA. Also, our results indicate the background is important in callus intuition and shoot formation from another culture in pomegranate.

Table 4. Effect of different concentrations of IBA on roots induction and growth of *Punica granatum*.

IBA (mg/l)	Root number	Root length (cm)
0.0	0.0[c]	0.0[b]
0.5	2.0[b]	3.0[a]
0.75	4.0 [b]	4.5[a]
1.0	7.0 [a]	2.5[a]

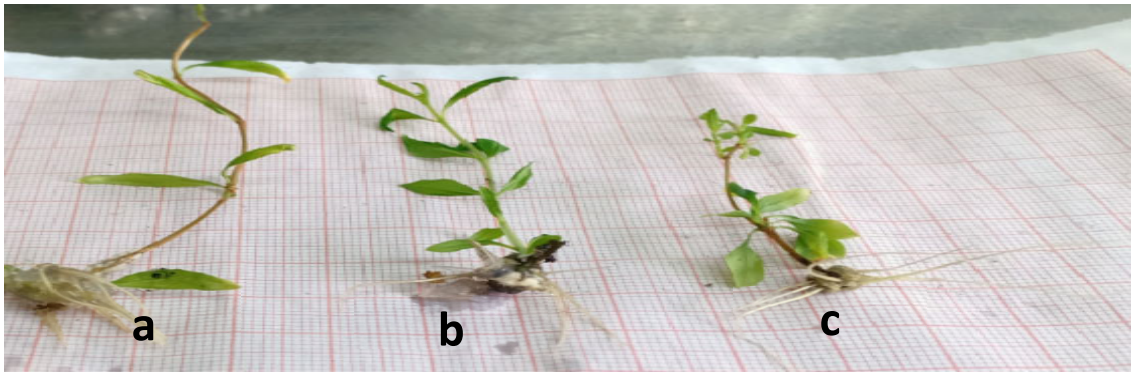

Fig. 3. Effect of different concentrations of IBA on percentage of roots initiation and shoots growth of pomegranate (a) 0.5 mg /l , (b) 0.75 mg/l and (c) 1 mg/l of IBA.

3. Conclusion

We can conclude from our result the important role played by chemicals, especially plant growth regulators, in the development and differentiation of cells from anther culture in pomegranate. The highest value of percentage of callus induction was on MS medium supplemented with 1 mg l of NAA and 2 mg l of BA. And our results indicate the background is important in callus intition and shoot formation from anther culture in pomegranate.

4. Experimental

4.1 Materials and methods

This work was conducted in the pomegranate block of the farm of Shandaweel Island Research Station, Sohag Governorate, Egypt and the laboratory of Fruit and Ornamentals Breeding Department and Biotechnology Research lab, Horticulture Research Institute. The four used plant growth regulators were purchased from Sigma-Aldrich (France).

4.2 Preparation and sterilization of explants:

The unopen flowers of the pomegranate cultivar were collected from field grown plants. The collected unopen flowers were washed in the tip water for 30 min these flowers were surface sterilized in different concentrations of corolex (20, 30, 40 %) for 20 min, these explants transferred to laminar flow hood the flower were disinfected with 70% ethanol three minutes and finally rinsed in sterile distilled water 3 to 5 times before culturing on the cultured medium.

Callus induction

Anthers can be extracted from unopened flowers that have previously been sterilized and were cultured on callus induction medium MS medium supplemented with different concentration of 2.4 D only and different combinations among different concentrations of (NAA and BA) (**Table 5**) and 30 g/l sucrose and 7 g/l of agar pH adjusted to 5.7 before autoclaved ten anther were culture in each jar (as one replicate) five replicates were used in this experiment for each treatment.

Table 5. MS medium supplemented with different growth regulator used callus induction from anther culture of pomegranate.

Medium code	Growth regulator mg/L		
	2.4.D	BA	NAA
MS1	1	-	-
MS2	3	-	-
MS3	5	-	-
MS4	-	2	2
MS5	-	2	1
MS6	-	4	0.5

The jars were incubated at 25 C under darkness for 45 day after 45 day from incubation period the following data were recorded for all experiments:

- Survival (%).
- Callus formation (%).

4.3 Differentiation stage.

The part of proliferated callus from the previous experiment was cultured on the differentiation media under fluorescent light (16 h/day). The callus were cultured on MS medium supplemented with different combinations of BA and NAA as BA at 1.0, 2.0 and 3.0 mg/l with NAA at 1.0, 2.0 and 3.0 mg/l. (**Table 6**). Three paces of callus (callus weight 0.5 to 1.0g) were cultured in each jar (as one replicate), five replicates were used in this experiment for each treatment.

The jars were incubated at 25^{O} C under fluorescent light (16 h/day) for 4 weeks after 4 weeks from incubation period the following data were recorded for all experiments:

-Shoot formation.(%)
-Shoot number .
-Shoot length (cm)

Explants were cultured on initiation medium and after four weeks the multiplicity shoots were transfer to multiplication medium for 3 subcultures.

Table 6. Different combinations between BA and NAA for callus differentiation of pomegranate through anther explants.

Medium code	Growth regulator mg/L	
	BA	NAA
MS1	1	1
MS2	1	2
MS3	1	3
MS4	2	1
MS5	2	2
MS6	2	3
MS7	3	1
MS8	3	2
MS9	3	3

4.4 Rooting stage.

Pomegranate in vitro shoots were transferred to MS medium supplemented with 30g/l sucrose, 7 g/l agar and different concentrations of IBA (0.5, 0.75 and 1 mg /l) and following parameters were recorded after 30 days.

The parameter:

a- The survival rate% $= \frac{(Total\ number\ survival\ plantletes)}{Total\ number\ of\ treated\ \ plantlet's} X100$

b- Number of roots

c- Length of root (cm)

Statistical analysis

The experiments for each stage were carried three time with five replicates, statistically analyzed by using analysis of variance.[24] Mean were differentiated by Duncan,s multiple range test at 5% level.[25] This work gives a great evidence that the applied sciences are very useful in different fields and this is reported also in a lot of research papers that published before.[26-59]

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