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Establishment of *in vitro* Micropropagation from Explants of *Punica granatum* L.

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Abstract: **The purpose of this study was to examine the ability of *in vitro* propagation of *Punica granatum* L. through tissue culture system. The experimental design that was used in this research is Completely Randomized Design (CRD). Cultures were stored at a temperature of 25 ± 1ºC and light 16 hours of light, 8 hours dark. All data was recorded and analyzed using ANNOVA. Leaf explants were found to be very responsive when cultured on MS medium supplemented with plant growth regulators at concentration of 2.0 mg/L Benzylaminopurine (BAP) + 1.5 mg/L Napthalene Acetic Acid (NAA) and has been identified as the optimum medium for shoot regeneration. Leaf explant produced 1.33±0.32 shoots per explant at week 8. Whereas from stem explant, Murashige and Skoog (MS) medium added with 0.5 mg/L BAP and 2.0 mg/L NAA gave the highest shoot development with 1.00 ± 0.28 shoots per explant Various combinations of auxin such as NAA and cytokinin such as BAP, at 1.5 mg/L was used for a complete regeneration of *Punica granatum* L. *in vitro*. In conclusion, *in vitro* micropropagation technique is a suitable alternative in producing new generation of *Punica granatum* L. and its features are preserved. It proves that plant tissue culture technology could be an alternative solution to achieve high quality of *Punica granatum* L., therefore could increase the crop production.**

Keywords: ***Punica granatum*** **L., tissue culture, micropropagation, explants, MS medium, growth regulators.**

I. INTRODUCTION

Micropropagation is the art and science of plant multiplication *in vitro*. The procedure of micropropagation incorporates many strides, for example; stock plant care, explant selection and sanitization, media control to get expansion, establishment acclimation, and development of the liners. Other than that, micropropagation is the act of quickly increasing the stock plant material to deliver countless plants, utilizing present day plant tissue culture strategies. Micropropagation is utilized to multiply new plants, for example; those that have been hereditarily changed or reproduced through ordinary plant rearing strategies. It is additionally used to give an adequate number of plantlets for planting from a stock plant which does not deliver seeds, or does not react well to vegetative multiplication.

The common propagation method of *Punica granatum* L. was by seed. However, germination by seeds shows a high degree of variability because of cross-pollinated nature of the plant. Other than that, the seeds of *Punica granatum* L. take time to germinate by using traditional method. This is because the seeds have hard seed coat, therefore, longer time to break the seed coat. Thus, it is not attractive approach for producing a large number of elite plants within a short period of time. Besides, the percentage of seed germination through conventional method is lower than through culture tissue technique. The demand of pomegranate is increasing due to its taste and medicinal properties. It is conventionally propagated by air layering and cuttings [1], [2] and [3]. In the present study, *in vitro* germination and regeneration of *Punica granatum* L. was conducted. The effects of plant growth regulators on *in vitro* micropropagation of explants of *Punica granatum* L. was observed. This study would help in overcoming the problem in propagation of pomegranate through conventional methods. A number of studies have been conducted on micropropagation of pomegranate by previous researchers [4] and [5] by using various types of explants including leaf, cotyledon, shoot tips and nodal explants.

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II. METHODOLOGY

A. Sterilization Seeds of Punica gratanum L.

Punica granatum L. seeds were surface disinfected before it was being used, prior to germination from intact seeds. Before the seed disinfection process, the juicy pulp was expelled from the seeds and the seeds were sun dried. The seeds required 8 hours to be dried. Seeds of *Punica granatum* L. were placed under running tap water for one hour followed by treatment with a few drops of tween 20 to dispose of undesirable debris and were washed 5-6 times using tap water. The seeds were then surface sterilized by treating them with 50% antiseptic germicide for 5 minutes followed by 40% sodium hypochlorite for 5 minutes and then washed again by using 15% sodium hypochlorite for 10 minutes. Subsequently, the seeds were washed three times with sterilized distilled water. Finally, the seeds were rinsed with 70% ethanol for 30 seconds and final rinsing with sterilized distilled water. Seeds were kept in sterilized distilled water for 24 hours in order to soften the seed coat.

B. Initiation Culture of Aseptic Seedlings

The zygotic embryo was extracted by making a flat cut on the wide part of the seed coat and placed on the MS [6] basal medium. All cultures were incubated at 25°C ±2 °C under 16-hour light photoperiod for germination. Thirty-day-old aseptic seedlings of *Punica granatum* L. were used as the source of explant.

C. Effects of Plant Growth Regulators on Punica gratanum L.

The explants obtained from *in vitro* seedlings of pomegranate was cut into 1.0 cm in length and cultured on MS medium supplemented with various concentrations of plant growth regulators, Benzylaminopurine (BAP) and Napthalene Acetic Acid (NAA) at the range of 0.5-2.0 mg/L. *In vitro* regeneration responses were investigated in this study. The culture medium contained MS medium (4.40 g/L basal salt blend), 30.0 g/L sucrose, 8.0 g/L technical agar and supplemented with different combinations of plant growth regulators. All refined media were adjusted to pH 5.8 before autoclaving at 121 °C for 20 minutes. All cultures were incubated under the cool white fluorescent lights at 25°C ±2°C under a 16-hour light and 8-hour dark photoperiod. Observation was done weekly.

III. STATISCAL ANALYSIS

Data in this study was recorded and analyzed. All data obtained was statistically analyzed. The data gathered from the experiments was analyzed according to mean percentages and analysis variance (ANOVA) at 5% level of significance. Each treatment was replicated 3 times with 30 explants for each replicate.

IV. RESULTS AND DISCUSSION

Micropropagation and development of shoots from the explants were observed in this study. The highest number of shoots development was 1.33 ± 0.32 from leaf explant when cultured on MS medium with 2.0 mg/L BAP and 1.5 mg/L NAA (Fig. 1), followed by 0.53 ± 0.23 on MS medium supplemented with 0.5 mg/L BAP + 2.0 mg/L NAA and the least shoot formation was observed when explants were cultured on MS media added with 1.0 mg/L BAP and 2.0 mg/L NAA with 0.13 ± 0.38 shoots per explant (Table 1). Whereas for stem explant, MS medium added with 0.5 mg/L BAP and 2.0 mg/L NAA gave the highest shoot development with 1.00 ± 0.28 shoots per explant (Fig. N). This followed by, MS medium added with 1.0 mg/L BAP with 2.0 mg/L NAA with 0.80 ± 0.29 shoots per explant and the least was MS medium with 1.5 mg/L BAP and 2.0 mg/L NAA with 0.26± 0.15 shoots per explant. However, root explant did not respond in all treatments.

Table 1: *In vitro* micropropagation of shoots from leaf and stem explants of *Punica granatum* L. cultured on MS medium supplemented with various concentration of BAP and NAA

Medium	Shoots per explant (Min±SE)	
	Leaf	Stem
MS media + 0.5 mg/L BAP + 2.0 mg/L NAA	0.53 ± 0.23b	1.00 ± 0.28a
MS media + 1.0 mg/L BAP + 2.0 mg/L NAA	0.13 ± 0.38d	0.80± 0.29b
MS media + 1.5 mg/L BAP + 2.0 mg/L NAA	0.33 ± 0.19c	0.26 ± 0.15d
MS media + 2.0 mg/L BAP + 1.5 mg/L NAA	1.33 ± 0.32a	0.73 ± 0.24b
MS media + 2.0 mg/L BAP + 2.0 mg/L NAA	0.466± 0.17b	0.36 ± 0.16c

Mean±SE, n=30. Mean with Different Letters in the Same Column Differ Significantly at p=0.05.

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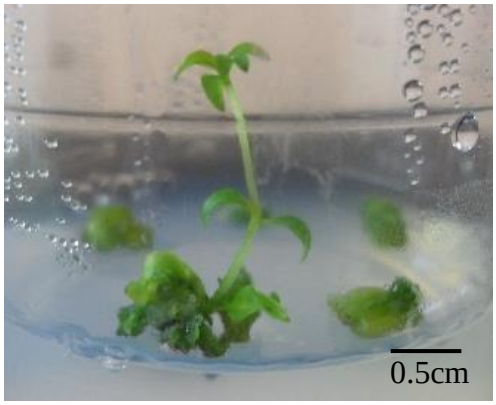

Fig 1: Optimum regeneration of shoots from leaf explants

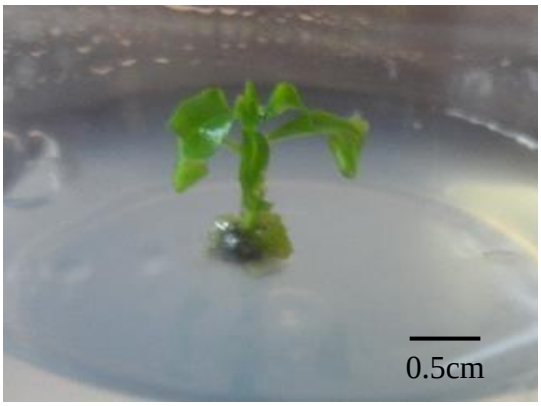

Fig 2: Optimum regeneration of shoots from stem explants

The research was done to investigate *in vitro* micropropagation responses from *Punica granatum* L. Tissue culture studies of *Punica granatum* L. showed that *in vitro* technique can be applied to this plant with high medicinal values, which is woody flowering plant as well. [7] discovered that *in vitro* techniques are considered as easy and reliable methods for rapid propagation of plants, especially medicinally important plants. In addition, in this study, the initiation of culture was carried out using aseptic seedlings. Advantages of using aseptic seedlings are, they were consisting of young or juvenile tissues which were actively dividing from meristematic cells and they are usually more responsive in culture.

The study on *in vitro* regeneration of *Punica granatum* L. was done through germination of aseptic seedlings as source of explant. Through this research, germination rate via traditional method was different through using tissues culture methods. Conventional breeding techniques of woody fruit trees is often difficult and slow because of high levels of heterozygosity and the long generation time between successive crosses as reviewed from time to time [8]. These difficulties necessitate the development of rapid and efficient regeneration protocol for *in vitro* propagation of elite genotypes. The various factors were studied to establish well regeneration system for this species. Factors studied in this study include type of explants and effect of hormones in order to obtain optimum regeneration system for *Punica granatum* L. The basic medium used in this study was [6] (MS medium).

Culture medium is one of the most important components of plant cell and tissue culture methods, so it is important to identify the suitable medium before *in vitro* work could be done further. Thus, MS medium was selected because it is the basic and generally used by researcher to begin their experiment. In addition, [9] stated that most noteworthy effectiveness of shoot arrangement utilizing cotyledonary leaf, acquired in MS medium containing 5.0 mg/L BAP and 0.2 mg/L NAA in *Ocimum bacilicum* L. In an alternate review, [10] demonstrated that among the distinctive fixations and mixes of development controllers, the most elevated rate of shoot arrangement (90%) and the most noteworthy normal number of shoots (5.88%) were seen in 0.2mg/L BAP from shoot tip explants of pomegranate. [11] stated that micropropagation of pomegranate was successful when cotyledon explants were cultured on MS medium supplemented with 1.0mg/L IBA + 0.1mg/L NAA.

In this research, regeneration of shoots was also obtained when leaf and stem explants were cultured on MS medium supplemented with various concentrations of BAP and NAA. A high recurrence of shoot organogenesis from leaf inferred callus of pomegranate was accomplished on MS medium supplemented with 1.0 mg/L BAP [12]. The shoot separation was obtained in the cotyledon determined callus of *Punica granatum L*. cv. Ganesh on MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L NAA [13].

In this study, micropropagation through stem explants was found to be successful because it had the highest shoot formation on MS medium supplemented with 0.5 mg/L BAP and 1.5 mg/L NAA. Formation of shoots was better achieved from stem than leaf and root. The length of young flower stalks or stem, which is related to the physiological age, influenced the percentage of adventitious bud formation. [14] showed that the position of stem explant has a great influence on the regeneration ability. In multiple shoot proliferation nodular explants response better than other explants via, leaves and inter nodes for this reason all experiments were carried out using nodal segments. Similar finding of auxiliary buds proliferation have also been reported in many medicinal plants [15] and [16]. The best response with maximum shoot elongation was obtained using 1.0 mg/L BAP in combination with 0.5 mg/L IAA after four weeks of culture. In this study, establishment of *in vitro* regeneration of shoots of *Punica granatum* L. was successfully achieved when leaf and stem were used as source of explant. Leaf explant gave optimum shoot regeneration. Meanwhile, root explant was found to be non-responsive in all treatments tested.

V. CONCLUSION

Tissue culture technique is the best alternative method to increase the germination rate of *Punica granatum* L. seeds and it *in vitro* micropropagation. Through the findings of this study, micropropagation and shoots regeneration of *Punica granatum* L. were successfully achieved. It is noticed that, tissues culture technique was a success and safe to use in order to achieve mass propagation of *Punica granatum* L. as well as other important crops. The findings of this study could be useful for further research on micropropagation of *Punica granatum* L. in future. Moreover, a complete protocol could be established as source of information for the *in vitro* micropropagation studies of this specific plant species in future. Therefore, mass propagation of *Punica granatum* L. could be achieved and the demand of this crop could be easily supplied.

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