The Bioscan 9(3): 1031-1035, 2014
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

FACTORS INFLUENCING *IN VITRO* GROWTH AND SHOOT MULTIPLICATION OF POMEGRANATE

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KEYWORDS
Multiplication
Adenine sulphate
pH
Shoots and light intensity

Received on :
29.10.2013

Accepted on :
10.07.2014

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ABSTRACT
Maximum proliferation of shoot (78.25 %), number of shoot (3.75) per explants and shoot length (3.06 cm) were obtained on MS medium supplemented with 1. 0 m g/L B A P + 1. 0 mg/L kinetin with 40 mg/L adenine sulphate in medium. Significantly higher number of shoots (4.5) and maximum length (3.38 cm) of shoot were recorded in 3 per cent sucrose. Maximum multiplication of shoot and length of shoot was observed at 3 per cent sucrose. Maximum number of shoots/explants (4.80) and shoot length (3.50 cm) was obtained under high (3000 lux) light intensity. Higher number of shoots per explant (4.25) and maximum length of shoot (3.70 cm) were recorded with pH 5.8. Multiplication and growth of shoots were significantly influenced by the different level adenine sulphate, sucrose, light intensity and media pH.

INTRODUCTION

Pomegranate (*Punica granatum* L.) belongs to the family punicaceae. It is native from Iran and spread throughout the Mediterranean region of Asia, Africa and Europe. India is one of the major pomegranates producing country. Pomegranate has great adaptability to saline soil and drought condition (Sepulveda *et al.*, 2000). It is commercially cultivated for its delicious fruits. A fully matured fruit contains many of the important nutrients and minerals and important constituent of protein, fat, fiber, carbohydrate etc. The fruit are rich in Fe, Ca, and antioxidant component like phenol, pigments and tannins. Fruit rind and bark of stem and root are widely used for tannin. It is used for treating dyspepsia and considered beneficial in treating leprosy. The conventional method of pomegranate propagation is hardwood cutting and layering. The conventional propagation methods of pomegranate are not always suitable for large-scale cultivation due to less availability of planting material at a time. *In vitro* techniques are one of the reliable sources used for commercial plantlet production of pomegranate. Various attempts have been made to multiply pomegranate by using tissue culture techniques through shoot tip and nodal segment explant of mature plant (Sharon and Sinha, 2000; Murkute *et al.*, 2004; Singh and Khawale, 2006; Chaugule *et al.*, 2007; Samir *et al.*, 2009; Kanwar *et al.*, 2009). The growth and multiplication of shoots in *in vitro* condition are affected by many factors (Anwar *et al.*, 2005). The effect of adenine sulphate is known in the tissue cultures at many plant species. Concentration and type of exogenous carbon sources into medium serve as energy and also to maintain the osmotic potential (Lipavska and Konradova, 2004). In general sucrose is a mostly useable carbon source for *in vitro* plant culture. Probably it is the most common carbohydrate in the phloem sap of many plants (Fuents *et al.*, 2000). Light is the ultimate substrate for photosynthetic energy conversion, higher light intensity causes photo oxidation which involves the destruction of chlorophyll. The effect of light intensity on *in vitro* propagation has attested by (Soontornchainaksaeng *et al.*, 2001) in orchid, (Jo *et al.*, 2008) in *Alocasia amazonica* and (Patel *et al.*, 2009) in Furcraea. Level of pH in media is also effect the growth and development of plantlets (Pierik, 1997). Till date, there is no such information available on the effect of adenine sulphate, sucrose, light intensity and level of media pH on shoot proliferation in pomegranate. Hence, the present investigation was carried out to study the influence of these factors on shoot growth and multiplication of pomegranate cv. Ganesh through nodal segment explant.

MATERIALS AND METHODS

Plant material and explant preparation

Trail was conducted using nodal segment explants from newly emerged shoots, containing one node each were collected from 4-5 years mature mother plant of pomegranate cv. Ganesh from Horticulture nursery, ASPEE College of Horticulture and Forestry, Navsari Agricultural University Gujarat. Leaves were removed leaving the petiole. They were swabbed with cotton dipped in 70 per cent alcohol and washed thoroughly in running tap water for about 20 minutes to remove traces of alcohol and dirt. The nodal segments were kept in a solution of 0.2 per cent bavistin (Carbendazim 50 per cent WP) for

one hour and triple washed with sterilized double glass distilled water then, they were sterilized with 0.1 % $HgCl_2$ (mercuric chloride) for 5 min and rinsed thrice with autoclaved distilled water under laminar air flow cabinet. The sterilized nodal segments were then cut and trimmed into small nodal explants of 2-3 cm length and then, they were quickly inoculated into medium.

Culture media and culture condition

For the establishment stage explants were inoculated on MS (Murashige, T. and Skoog, F., 1962) medium supplemented with 1.0 mg/L BAP + 0.5 mg/L NAA and 0.8 % agar. Media were autoclaved at 121ºC and 15 Ib/in^2 for 20 min. From the established culture about 1-2 cm long proliferated shoots were isolated and transfer to MS medium fortified with BAP (0.5-1.5 mg/L) in combination with kinetin (0.5-1.0 mg/L).The Effect of six levels of adenine sulphate (20, 30, 40, 60, 80, and 100 mg/L) were tested in first batch of culture. In another batch of culture six levels of sucrose (1, 2, 3, 4, 5 and 6 %) were supplemented in the same media composition at pH 5.8. The effect of light intensity were examined by keeping the culture into four different (1000, 2000, 3000 and 4000 lux) light intensity, provided by white fluorescent tube of 36 watt, kept 50 cm above bench surface. Six levels of pH (4.5, 5.0, 5.5, 5.7, 5.8 and 6.0) were tested for influence of pH on shoot proliferation (Mishra, 2011).The pH of media was adjusted prior to autoclaving. All the cultures were incubated in a culture room at a temperature of 26 ± 2ºC with relative humidity at 55 ± 5 per cent. The cultures were maintained in 16/8 h light/dark period for three week.

Statistical analysis

All the experiments were setup in the completely randomized design and repeated three times, each treatment consisted of 50 explants and the means separation were done according to Least Significant Differences (LSD) at 5% level.

Table 1: Effect of adenine sulphate on *in vitro* shoot multiplication and growth of pomegranate cv.Ganesh

Adenine sulphate (mg/L)	Proliferation of shoot (%)	No. of Shoot/ culture	Length of shoot (cm)
20	17.50 (24.72)*	1.67	1.33
30	28.00 (31.94)	2	1.68
40	78.25 (62.20)	3.75	3.06
60	61.00 (51.36)	2.92	2.35
80	28.50 (32.27)	1.65	1
100	24.45 (29.48)	1.29	0.65
S.Em. ±	0.45	0.15	0.1
CD at 5 %	1.34	0.46	0.29

* Figure in parentheses is arcsine transformed value

RESULTS AND DISCUSSION

Effect of adenine sulphate on shoot growth and multiplication

Proliferation and growth of shoots were significantly influenced by the concentration of adenine sulphate in the medium. Maximum proliferation of shoot (78.25 %), number of shoot (3.75) per culture and shoot length (3.06 cm) were obtained at the concentration 40 mg/L adenine sulphate in MS medium supplemented with 1.0 mg/L BAP + 1.0 mg/L kinetin after 3-4 week of culture (Table 1; Fig. 3a). These findings are in conformity with the (El-Shamy, 2002) in Bougainvillea, (Singh and Khawale, 2006) in pomegranate, (Bantawa *et al.*, 2009) in *Picrorhiza scrophulariiflora* and (Gatica *et al.*, 2010) in common bean. Adenine sulphate can boost cell growth and greatly enhance the shoot formation, as it is the additional source of nitrogen to cells, which can be taken up more rapidly than inorganic nitrogen (Harry and Thorpe, 1994). The role of adenine sulphate on shoot proliferation was found more effective when it combined with cytokinins (Staden *et al.*, 2008). In the present study the effect of adenine sulphate was shows positive response up to 40 mg/L for increasing shoot growth and then, declined the shoot growth (Table 1). Concentrations above 60 mg/L resulted in a reduced number of nodes, number of lateral branches and discoloration of leaf. Addition of higher concentration of adenine sulphate in the medium does not always help to shoot growth. It may be due to the unbalance of indigenous hormonal level in culture with higher level of adenine sulphate. The declined trend in shoot growth due to the addition of more adenine sulphate was also reported by (Al-Sulaiman, 2010) in *Ziziphus spina-christi*.

Effect of Light intensity on shoot growth and multiplication

Various level of light intensity were tested by keeping the constant temperature 26 ± 2ºC and 16/8 h light/dark period in the incubation room. It is evident from the (Table 2) that shoot multiplication was significantly influenced by different light intensity. The maximum number of shoots (4.8) per explants and highest length of shoot (3.50 cm) was recorded under 3000 lux light intensity, while minimum shoot (0.85 cm) length recorded under 1000 lux light intensity. Similarly, maximum number of shoots/culture and healthy shoots were observed at 3000 lux, as against 1000 lux light intensity (Fig. 3b). The results are clearly indicated that light intensity in the range of 1000 - 3000 lux showed gradually increasing in the shoot growth. While, in the rage of 4000 lux it showed decline in the growth and yellowing of the shoots. This might be due to the interaction between light intensity and internal factors

Table 2: Effect of different light intensities on *in vitro* shoot multiplication and growth of pomegranate cv. Ganesh

Light intensity (Lux)	Growth rate of shoot	No. of shoot/explant	Length of shoot (cm)	Remark
1000	+	1	0.85	Stunted growth
2000	+ +	2	1.8	Defoliation occurred
3000	+ + +	4.8	3.5	Good growth
4000	+ + +	1.8	1.83	Yellowing of shoots
S.Em. ±	-	0.13	0.15	
CD at 5%	-	0.4	0.48	

+ Little growth, + + Medium growth, + + + Better growth

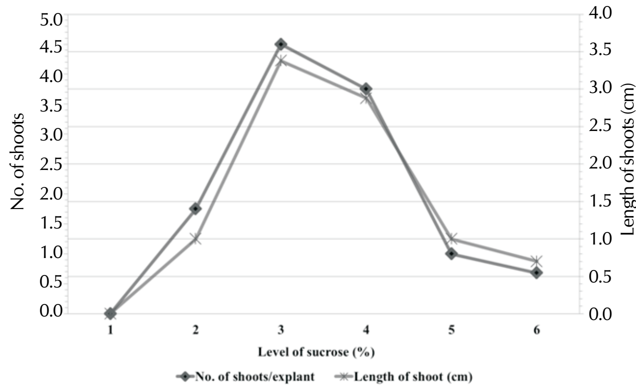

Figure 1: Effect of different level of sucrose on *in vitro* shoot multiplication of pomegranate cv. Ganesh

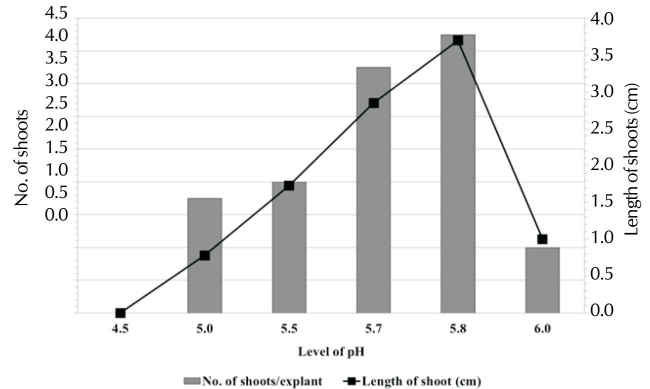

Figure 2: Effect of initial pH of the medium on *in vitro* shoot multiplication of pomegranate cv. Ganesh

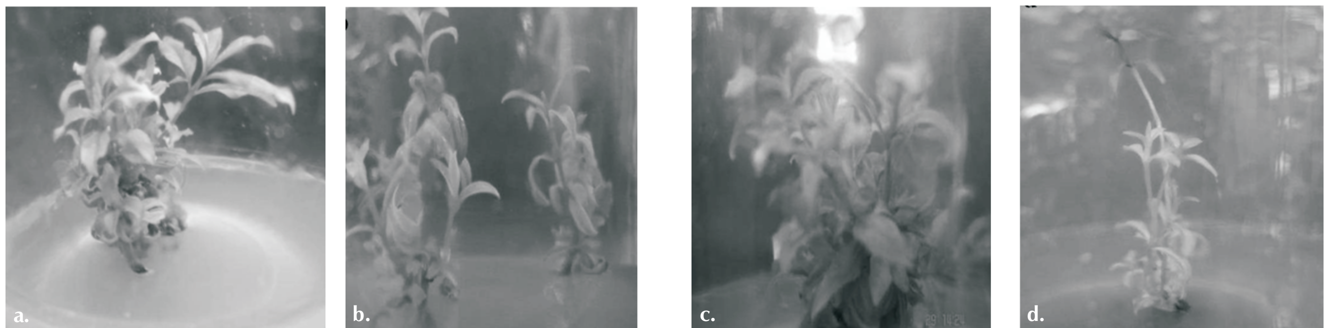

Figure 3(a-d): Proliferation of shoots in MS medium containing 1.0 mg/L BAP + 1.0 mg/L Kinetin with 40 mg/L adenine sulphate (a), Proliferation of shoot at 3000 lux light intensity (b), Shoot multiplication at 3 % sucrose in medium (c), Shoot proliferation at 5.8 pH of medium (d).

which directly affect plant growth. These results are coinciding with those obtained by Patel (2008) in papaya and Shinde (2008) in grape. Light intensity exhibited more effective role on the multiplication of shoots in pomegranate (Sharon and Sinha, 2000) and (Singh and Khawale, 2006). Light is an ultimate substrate for photosynthetic energy conversion. Though, higher light intensity causes photo oxidation which involves the destruction of chlorophyll, resulting in less biomass production and yellowing of shoot. High light intensity is damaging to the water-splitting photosystem II (PSII), (Soontornchainaksaeng *et al.,* 2001) leading to degradation of the reaction centre resulting reduction in growth and discoloration of leaf.

Effect of sucrose level on shoot growth and multiplication

Sucrose levels in the medium were found to be the most effective on the proliferation and growth of shoots. Sucrose is usually the carbohydrates of choice in most of the cell culture media. Attempts were made to find out optimum concentration of sucrose as a carbon source for *in vitro* establishment and growth of explants. Among all the treatments tested significantly higher number of shoots (4.5) per culture and maximum length (3.38 cm) of shoot were recorded on MS medium containing 1.0mg/L B A P + 1.0 mg/L kinetin with 3 per cent sucrose added in medium (Fig 1). It is apparently seen that multiplication of shoot and length of shoot was increased with the increased in sucrose level up to 3 per cent. This may be due to the higher concentration of sugar levels in the culture medium which caused increased cell division vis-à-vis increase in the volume of the tissue (Gurel and Gulsen, 1998). Similar results were reported by Kumar and Kumar (1998) in pomegranate, Anjum and Pandey (2011) in *Annona reticulate* (L.) and (Sharma *et al.,* 2012) in Kinnow. From the present results it was observed that out of six levels of sucrose tested, 3 per cent sucrose gave high shoot proliferation and length of shoot and then, there was decline trend in the growth at 4 per cent sucrose level (Fig, 3c). Shinde (2008) also observed the declined trends in shoot growth at 4-5 per cent sucrose level in the MS medium containing 2.0 mg/L BAP + 0.1 mg/L NAA in grape. Sucrose plays an important role to maintain the osmotic potential and conservation of water in cells. However, higher concentration of carbohydrates in the plant may lead to decline in osmotic potential of the cell, resulting loss of chlorophyll (Jain and Babbar, 2003). This leads to discoloration of leaf and reduction in shoot growth (Yaseen *et al.*, 2009).

Effect of media pH on shoot growth and multiplication

The data on influence of initial pH of the medium on shoot multiplication and growth are presented in (Fig 2). Higher number of shoots per culture (4.25) and maximum length of shoot (3.70 cm) were recorded with pH 5.8. Among all the treatment, the trends in number of shoots per explant and length of shoot increased as pH level increased up to 5.8, and then, decreased the growth. Maximum multiplication rate and best shoot growth was observed at pH 5.8 (Fig.3d) While, pH level 4.5 did not produce any shoot from the culture.

Moreover, the growth of shoot and multiplication rate was decreased at lower 4.5-5.5 as well as higher 6.0 pH level. Similar results were observed by (Patel *et al.,* 2009) in Furcraea and Shinde (2008) in grape. The influence of pH in present investigation might be due to the well known effect of pH on the availability of nutrients from the medium. The pH of medium influences the uptake of nutrients (Owen *et al.,* 1991) and also availability of plant growth regulators (Arya *et al.,* 2012). Effect of pH on the availability of nutrients in the culture medium was reported by (Hurley *et al.*, 1981). According to Street, (1966), the ambient pH could be desire for absorption of various nitrogen sources; pH growth response curves indicated that nitrate N when pH is acidic (4.7-4.9 pH approx.), ammonical N at neutral pH (7.0-7.2 pH approx.) and nitrate at pH 5.0 to 6.0 supported maximum growth. The result obtained with in present investigation may be considered in light of presence of nutrients for growth, at pH 5.8 suggesting favorable effect of nitrate N rather than ammonical nitrogen.

ACKNOWLEDGEMENT

The authors are thankful to University Department of Biotechnology and Dean, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, Navsari, Gujarat for providing lab facilities to carry out the investigation.

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