Effect of Culture Media, Gelling Agents and Plant Growth Regulators on the Regeneration of Dwarf Pomegranate (*Punica granatum* var. *nana*)©

Shingo Terakami, Yoshihiko Sekozawa, Sumiko Sugaya, and Hiroshi Gemma
Tsukuba University, Tennoudai, Ibaraki 305-8572, Japan

Nagao Matsuta, Junnichi Soejima
National Institute of Fruit Tree Science, Fujimoto, Ibaraki 305-8605, Japan

INTRODUCTION

Perennial fruit crops are unable to flower or produce fruits for years from seedlings. This condition, lacking reproductive capability, is defined as juvenility. A long juvenile phase is serious constraint in traditional breeding and cultivation. Juvenility in perennial fruit crops is in need of greater research because being able to reduce the juvenile phase would lead to faster development of new cultivars in agricultural industries.

Dwarf pomegranate bears fruit within 1 year from seed, shows perpetual growth in a greenhouse, and shoot regeneration occurs easily (Omura et al., 1987b). In pomegranate, many studies of shoot regeneration have been performed (Omura et al., 1987a; Moriguchi et al., 1987; Jaidka and Mehra, 1986; Naik et al., 1999; 2000).

To elucidate juvenility, dwarf pomegranate seems to be a useful model species by using gene transfer technique. *Agrobacterium tumefaciens* is a gram-negative soil bacterium and is capable of transferring T-DNA. Transferred T-DNA is imported into the nucleus and integrated into plant chromosome. *Agrobacterium*-mediated transformation of fruit trees has been reported in apple (Zhu et al., 2001), pear (Mourgues et al., 1996), grape (Torregrosa et al., 2002), and citrus (Weliton et al., 2003). The breeding of fruit trees requires many labors and large areas for screening because of their long vegetative phase. Gene transfer technique is a powerful tool for the introduction of foreign genes into fruit trees and can shorten the time for breeding. In fact, this method was applied to breeding in papaya (Tennant et al., 2001).

Regardless of the gene transfer technique, its use is limited due mostly to a shortage of an efficient regeneration system. Therefore, our objective in this study was to develop a regeneration protocol for dwarf pomegranate from leaf segments as affected by different culture media, gelling agents, and plant growth regulators.

MATERIALS AND METHODS

Dwarf pomegranate (*Punica granatum* L. var. *nana*) grown in the greenhouse of the National Institute of Fruit Tree Science located in Tsukuba, Japan, was used. With a few modifications, the protocol of Omura et al. (1987b) was used to regenerate shoots from young leaf segments. Young leaves were sterilized with 0.25% sodium hypochlorite for 3 min, rinsed 5 times in sterilized distilled water, cut into fragments about 3 mm in length, and placed on the regeneration medium with the adaxial surface adhering to the medium. To test the effect of culture media and gelling agents on the regeneration rate, B5 (Gamborg et al., 1968), MS (Murashige

Table 1. Effects of culture media and gelling agents on both the regeneration rate and the shoot number of dwarf pomegranate.

Culture media	Gelling agent	Gelling agent concentration (%)	Regeneration rate (%) (mean±S.E.)[a]	No. of shoots/ regenerating leaf (mean±S.E.)[a]
B5	Agar	0.4	0	0
		0.8	10.67±1.76	1.28±0.11
		1.2	0	0
	Gelrite	0.2	0	0
		0.3	0	0
MS	Agar	0.4	20.00±5.03	1.29±0.02
		0.8	24.00±1.15	1.30±0.07
		1.2	2.67±1.76	1.17±0.17
	Gelrite	0.2	6.67±2.40	1.62±0.22
		0.3	24.67±0.67	1.51±0.06
NN	Agar	0.4	23.33±5.46	1.35±0.11
		0.8	21.33±0.67	1.56±0.19
		1.2	0	0
	Gelrite	0.2	0	0
		0.3	0	0

[a]Each numerical value is the average of three replications in a set of experiments. Fifty segments of leaf were cultured in an experiment.

and Skoog, 1962), or NN (Nitsch and Nitsch, 1969) were used as basal media. They were supplemented with 0.5 μM α-naphthaleneacetic acid (NAA) and 5 μM N^6-benzyladenine (BA) with different solidified agents: agar with 0.4, 0.8, or 1.2% (WAKO, Japan) or Gelrite with 0.2% or 0.3% (WAKO, Japan) at pH 5.8. The explants were cultured at 25 ºC in the dark. Regeneration rate and the number of adventitious shoots were investigated after 80 days of culture.

Concomitantly, the following plant growth regulators added to MS medium solidified with 0.3% Gelrite were examined; NAA at 0, 0.1, 2.5, or 5.0 μM and with BA, kinetin, zeatin, 2-isopentenyladenine (2-iP) at 0, 5, 10, or 20 μM, N-(2-chloro-4-pyridyl)-N'-phenylurea (CPPU) or thidiazuron (TDZ) at 0, 0.5, 1.0, or 2.0 μM at pH 5.8. The explants were cultured at 25 ºC in the dark.

RESULTS

The adventitious shoot formation from young leaf of dwarf pomegranate was influenced by culture media and gelling agents and their concentration (Table 1). High frequency of shoot formation was observed at the cutting surface of the leaf segments after 80 days of culture on MS medium solidified with 0.3% Gelrite in the dark. In this study, the callus formation was observed on all medium, but varied in the timing of its appearance was dependent on medium. Calli were induced about 30 days after incubation on MS and NN medium. On B5 medium, callus formation was rapid and large, but calli easily turned brown. Significant differences in the number of shoots were not found among media.

We tested the combination of NAA and six different cytokinins at various concentrations to determine the optimal combination and concentration of the plant growth regulators (Table 2-6). The highest regeneration rate and number of shoots

Table 2. Effects of the combination of NAA and BA on both the regeneration rate and the shoot number of dwarf pomegranate on MS basal medium.

NAA (µM)	BA (µM)	Regeneration rate (%) (Mean±S.E.)[a]	No. shoots/ regenerating explant (Mean±S.E.)[a]
0	0	0	0
	5	3.33±0.67	1.00±0.00
	10	4.67±1.33	1.11±0.11
	20	1.33±0.67	0.67±0.33
0.5	0	0	0
	5	24.00±2.31	1.39±0.03
	10	44.67±3.71	1.72±0.02
	20	12.00±1.15	1.27±0.08
2.5	0	0	0
	5	8.67±1.76	1.56±0.06
	10	24.67±1.76	1.27±0.03
	20	13.33±0.67	1.46±0.11
5.0	0	0	0
	5	10.67±1.76	1.00±0.00
	10	20.00±1.15	1.23±0.09
	20	10.00±2.00	1.44±0.20

[a] Each numerical value is the average of three replications in a set of experiments. Forty segments of leaf were cultured in an experiment.

Table 3. Effects of the combination of NAA and kinetin on both the regeneration rate and the shoot number of dwarf pomegranate on MS basal medium.

NAA (µM)	Kinetin (µM)	Regeneration rate (%) (mean±S.E.)[a]	No. shoots/ regenerating explant (mean±S.E.)[a]
0	0	0	0
0	0	0	0
	5	0	0
	10	0	0
	20	0	0
0.5	0	0	0
	5	0	0
	10	0	0
	20	8.67±0.67	1.15±0.08
2.5	0	0	0
	5	0	0
	10	0	0
	20	6.67±1.33	1.00±0.00
5.0	0	0	0
	5	0	0
	10	0.67±0.67	0.33±0.33
	20	2.67±0.67	1.33±0.33

[a] Each numerical value is the average of three replications in a set of experiments. Forty segments of leaf were cultured in an experiment.

Table 4. Effects of the combination of NAA and 2-iP on both the regeneration rate and the shoot number of dwarf pomegranate on MS basal medium.

NAA (μM)	2-iP (μM)	Regeneration rate (%) (Mean±S.E.)[a]	No. shoots/ regenerating explant (Mean±S.E.)[a]
0	0	0	0
	5	0	0
	10	0	0
	20	0	0
0.5	0	0	0
	5	4.67±0.67	1.27±0.14
	10	5.33±0.67	1.11±0.11
	20	4.00±1.15	1.61±0.20
2.5	0	0	0
	5	6.67±0.67	1.41±0.12
	10	2.00±1.15	1.16±0.17
	20	0	0
5.0	0	0	0
	5	0	0
	10	0	0
	20	0	0

[a] Each numerical value is the average of three replications in a set of experiments. Forty segments of leaf were cultured in an experiment.

Table 5. Effects of the combination of NAA and N-(2-chloro -4-pyridyl)-N'-phenylurea (CPPU) on both the regeneration rate and the shoot number of dwarf pomegranate on Murashige and Skoog basal medium.

NAA (μM)	CPPU (μM)	Regeneration rate (%) (Mean±S.E.)[a]	No. shoots per regenerating explant (Mean±S.E.)[a]
0	0	0	0
	0.5	0	0
	1.0	0	0
	2.0	0	0
0.5	0	0	0
	0.5	4.67±0.67	1.44±0.06
	1.0	2.00±1.15	0.67±0.33
	2.0	0	0
2.5	0	0	0
	0.5	2.00±1.15	0.67±0.33
	1.0	1.33±1.33	0.33±0.33
	2.0	0	0
5.0	0	0	0
	0.5	2.67±0.67	1.50±0.29
	1.0	1.33±0.67	0.67±0.33
	2.0	0	0

[a] Each numerical value is the average of three replications in a set of experiments. Forty segments of leaf were cultured in an experiment.

Table 6. Effects of the combination of NAA and thidiazuron (TDZ) on both the regeneration rate and the shoot number of dwarf pomegranate on Murashige and Skoog basal medium.

NAA (μM)	TDZ (μM)	Regeneration rate (%) (Mean±S.E.)[a]	No. shoots per regenerating explant (Mean±S.E.)[a]
0	0	0	0
	0.5	0	0
	1.0	0	0
	2.0	0	0
0.5	0	0	0
	0.5	16.67±2.40	1.25±0.04
	1.0	5.33±1.33	1.25±0.14
	2.0	2.67±0.67	1.17±0.17
2.5	0	0	0
	0.5	10.00±0.00	1.13±0.07
	1.0	4.00±1.15	1.28±0.15
	2.0	3.33±1.33	1.00±0.00
5.0	0	0	0
	0.5	7.33±1.76	1.30±0.15
	1.0	0	0
	2.0	0	0

was produced on the medium supplemented with 0.5 μM NAA and 10 μM BA (Table 2). Effects of kinetin, 2-iP, CPPU, and TDZ on the regeneration of dwarf pomegranate are low at these concentrations. Adventitious shoot formation was not detected on the medium supplemented with zeatin and/or NAA (data not shown).

DISCUSSION

As reported by Omura et al. (1987b), the proper culture medium and gelling agent for shoot regeneration of dwarf pomegranate has been tested, but so far the combination of culture medium and gelling agent has not been investigated. Our result showed that MS medium solidified with 0.3% Gelrite effectively produced adventitious shoots. Microscopic observation indicated that adventitious shoots were regenerated from the calli formed at the cutting surface of the leaf segments. On B5 medium, callus formation was rapid and large, but many calli turned brown. These results are in agreement with a previous report by Omura et al. (1987b). Tissue browning caused a decrease in regeneration from callus in dwarf pomegranate and it is one of the serious problems associated with the low regeneration rate in some of woody plants. Browning is generally considered to result from the oxidation of phenolic substances released from the cutting surface of the explants. In previous studies (Welander and Maheswaran, 1992; Chevreau et al., 1997; Burgos and Alburquerque, 2003) gelling agents and their concentration affected regeneration rate, as shown with our results. When the concentrations of gelling agents (agar or Gelrite) were kept low, regeneration rate was low, although callus formation was clearly induced (data not shown). From these results, the medium solidified with gelling agent at low concentrations was not applicable to regeneration of dwarf pomegranate. Similarly, low concentrations of gelling agent promoted hyperhydricity in apple (Pasqualetto et al., 1986; 1988) and pear (Turner and Singha, 1990; Kadota et al., 2001).

In this study, BA promoted shoot regeneration from leaf segments of dwarf pomegranate and the highest regeneration rate, 44%, was obtained on MS medium containing 0.5 μM NAA and 10 μM BA. These results indicate that a combination of NAA and BA promotes shoot regeneration of dwarf pomegranate leaf explants and that addition of NAA as well as cytokinin is a critical component for regeneration of dwarf pomegranate. Previous studies in pear (Caboni et al., 1999), pomegranate (Naik et al., 2000), and *Ribes magellanicum* (Arena and Pastur, 1997) demonstrated that BA was more effective for regeneration than other cytokinins. We used TDZ and CPPU at high concentration (5, 10, or 20 μM) with NAA, but no callus formation was observed (data not shown). Adventitious shoots were induced by TDZ when cultured on the medium at low concentration, similar to previous reports, showing high cytokinin activity (Huetteman and Preece, 1993; Naik et al., 1999).

In conclusion, MS medium supplemented with 0.5 μM NAA, 10 μM BA, and 0.3% Gelrite is suitable for the regeneration from the leaf segments of dwarf pomegranate. Experiments are now in progress to investigate factors affecting *Agrobacterium*-mediated transformation and regeneration of transformed shoots. Our results seem to be effective for the transformation of dwarf pomegranate. We will study juvenility by the introduction of flowering genes, such as FLOWERING TIME and CONSTANS.

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