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Short Communication

Multiple Shoot Bud Formation and Plantlet Regeneration from *in vitro* Cultured *Pistacia vera* Seeds

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Abstract. Studies were carried out on the primordial initiation and development of shoot buds derived from pistachio (*Pistacia vera*) seedlings, cultured on MS medium with added 6-benzylaminopurin (BA). The *in vitro* culture of pistachio seeds in the presence of BA (1.0 - 4.0 mg/l), plus kinetin (Kin, 1.0 mg/l) and naphthalene acetic acid (NAA, 0.25-1.0 mg/l) stimulated varying degree of seed germination and the number of shoots produced. When excised single shoots from these *in vitro* cultured seeds were subcultured on fresh medium containing high concentration of BA (4.0 mg/l), alongwith Kin and NAA, multiple shoot production was observed. Normal bud growth and shoot elongation were achieved by transferring cultures to the MS medium containing low concentration of the growth regulators BA (1.0 mg/l), plus NAA (0.25 mg/l) and Kin (1.0 mg/l).

Keywords: *Pistacia vera*, pistachio plantlet regeneration, multiple shoot formation, *in vitro* seed culture, primordial initiation

Pistacia vera (the pistachio nut plant) is a tree crop, which grows naturally in the low rainfall areas where the irrigation water supply is also insufficient. The plant produces nuts, which are of significant economic importance having high nutritional value. Inspite of these attributes, arboriculturists are not sufficiently attracted to pistachio orchard growing. It is partly due to the imbalanced ratio of male and female plants and the late expression of plant sex, at the age of 6-7 years. The male plants far outnumber the female plants. Thus, the plant nurseries raised from seeds put a serious constraint on time and money in terms of investment on non-productive stock. In addition to this, the other serious causes that hinder the propagation of this plant include its lack of resistance to pests and some common diseases, as well as the instability of characters in the seed-grown plants. A rapid multiplication method, based on a disease resistant and stable character stock, is likely to help overcome these problems effectively.

The potential of tissue culture techniques for the propagation of woody plants has been extensively reported (Nowak *et al*., 2004; Quraishi *et al*., 2004; Ahmed *et al*., 1995; Philips, 1984; Zimmerman, 1979; Boxus, 1974; Mehra and Mehra, 1974). Although the pistachio tissue culture has also been reported, the success in plant regeneration has been only limited (Ahmed *et al*., 1995; Jabeen *et al*., 1995; Baragchi, 1986a; 1986b; Bustamante-Garcia, 1984). The present study reports the requirements for successful *in vitro* multiple shoot formation and plantlet regeneration from excised single juvenile shoots of pistachio raised from *in vitro* cultured seeds.

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Seeds of *Pistacia vera* were procured from the Department of Agriculture, Quetta, Pakistan. After removing the testa, the pistachio seeds were submerged in 95% ethanol for a few seconds and distinfected by immersion in 0.1% $HgCl_2$ solution containing 2-3 drops of Tween-20 per 100 ml of the disinfectant. After 20 min, the seeds were rinsed three times with sterile distilled water and placed directly on the MS medium (Murashige and Skoog, 1962). Growth regulators, such as 6-benzylaminopurin (BA), kinetin (Kin) and naphthalene acetic acid (NAA), were added to the defined MS medium individually (BA), or in combination (BA+NAA, NAA+ Kin, BA+NAA+Kin). To obtain the growth of regulator-treated seedlings, pistachio seeds were inoculated on the defined MS medium, supplemented with various concentrations and combinations of growth regulators. The growth medium pH was adjusted to 5.7. To the liquid MS medium were added 0.8% agar and 3% sucrose, which was then autoclaved for 20 min at 121 °C at 15 lb psi pressure. The pistachio seeds were aseptically placed on the solidified culture medium and incubated in a growth chamber at 20±2 °C with 16 h photoperiod. Cool white fluorescent light of 3000 Lux intensity was provided in the growth chamber.

The effect of addition of BA, alone, or of BA and Kin in combination with NAA on the pistachio seed germination was examined. The results showed that the percentage of seed germination was high (42-47%), whether BA was used alone, or when BA or Kin was used in combination with NAA (Table 1). The seed germination, in comparison, was below 30% when seeds were *in vitro* cultured on the MS basal medium with no added growth regulators. The rate of seed germination was

around 42%, when these were grown on the medium supplied with BA (1.0 mg/l) alone, or in combination with the auxin NAA (0.25 mg/l). Average dia of the stem shoots was 1.0 and 1.5 mm, and the leaf size (length x width) was 10x7 mm and 15x5 mm, respectively. However, no shoot proliferation was observed. The addition of Kin (1.0 mg/l), instead of BA in combination with NAA (0.25 mg/l), to the basal MS medium, slightly improved the rate of germination to 47%; furthermore, the average dia of the stem was also noted to increase to 2.0 mm and the leaf size to 25x15 mm. However, as with BA and BA+NAA, no shoot proliferation was noted. Seeds cultured on the medium containing higher concentration of BA (4.0 mg/l), in combination with NAA (1.00 mg/l) and Kin (1 mg/l), showed an increase in the rate of seed germination to 56% (Table 1). The seedlings were additionally observed to produce multiple shoots with the stem shoot dia 3.0 mm and leaf size 40x30 mm. These observations indicate that the growth regulator combination of BA+NAA+Kin resulted in stronger and multiple shoots in the *in vitro* cultured pistachio seedlings.

The *in vitro* cultured seedling gave rise to one main single shoot (Fig. 1), when the seeds were cultured on the MS medium containing low concentration of BA (1.0 mg/l) whether alone, or when used in combination with NAA (0.25 mg/l) or NAA in combination with Kin (0.25 mg/l + 1.0 mg/l, respectively). The root system in these seedlings usually consisted of a long root, which produced extensive lateral roots. In contrast to these observations, at the higher concentration of BA (4.0 mg/l), in the presence of Kin and NAA (1.0 mg/l and 1.0 mg/l, respectively), the dormant axillary buds present in the axil of cotyledonary stalks became active and produced multiple shoots at the base of the cotyledonary stalk attachment (Fig 2). The morphological development of shoot and root apices was also altered and multiple shoot formation was achieved (Table 1). The observed response of pistachio seeds cultured *in vitro* was similar to that described for both *in vitro* and *in vivo* systems reported for various other plants (Purohit *et al*., 2002; Polisetty *et al*., 1997; Bhatia *et al*., 1985). Hence, the developmental behaviour of seedlings cultured *in vitro* in the presence of high concentration of BA indicated a definite advantage for the use of excised shoot segments for the *in vitro* regenerative expression of multiple shoot bud formation. Conversely, the low concentration of BA was not effective in the stimulation of shoot bud proliferation, though the growth of the primary bud produced normal shoot as also reported by Nadgauda *et al.* (1978). It was further observed that the excised shoot segments, when subcultured, regenerated multiple shoots in the presence of high concentration of BA (4.0 mg/l), which continued to produce shoot buds without elongation.

Fig. 1. *Pistacia vera* seed cultured *in vitro* on growth medium supplementad with 6-benzylaminopurin (1.0 mg/l), and kinetin and naphthalene acetic acid (1.0 mg/l + 0.25 mg/l, respectively) producing single main shoot.

Fig. 2. *Pistacia vera* seed cultured *in vitro* on growth medium supplementad with 6-benzylaminopurin (4.0 mg/l), and naphthalene acetic acid and kinetin (1.0 mg/l + 1.0 mg/l, respectively), producing multiple shoots.

Table 1. The effect of growth regulators on the germination rate and seedling vigour

Combination of growth regulators (mg/l)			Germination (%)*	Shoot growth		Leaf size (mm)**
				thickness (dia, mm)*	number	
BA	NAA	Kin				
1.00	-	-	42.5±1.51	1.0±0.55	single	10x7
1.00	0.25	-	42.7± 1.92	1.5±0.72	single	15x5
-	0.25	1.00	47.5±0.99	2.0±0.68	single	25x15
4.00	1.00	1.00	55.9±1.95	3.0±0.36	multiple	40x30

*± standard deviation;**length x width; BA= 6-benzy-laminopurin; NAA= naphthalene acetic acid; Kin = kinetin

The pistachio shoot segments obtained from the seedlings cultured *in vitro* in the presence of various growth regulators, whether singly, or in combination at different concentrations, were subscultured for regeneration studies to optimise the conditions. The optimum conditions for obtaining multiple shoot formation in pistachio seedlings were determined as: (a) *in vitro* initiation from seeds inoculated on MS medium supplemented with growth regulators and auxin (BA, 4.0 mg/l + Kin, 1.0 mg/l + NAA, 1.0 mg/l) incubated for 4-6 weeks; (b) further stimulation of shoot buds for 4 weeks in fresh medium of the same composition as for (a); and (c) stimulation of rhizogenesis of shoots by the transfer of the cultures to fresh MS medium containg an effective auxin. Under these optimized conditions, more than 50 shoots per seed were generated within 9-11 weeks.

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